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13. SUPPLEMENTARY NOTES

14. ABSTRACT During this second year of the project, we established multiple primary cell strains from normal controls and SSc patients. We performed stimulation assays with silica in 82 primary fibroblast strains. Our results showed that silica activate fibroblasts toward fibrotic changes. However, different fibroblast strains obtained from different individuals showed different responses in terms of the gene expression of the ECM components. Using longitudinal linear models in analysis of association betweenspecific genotypes and dynamic changes of gene expression of the fibroblasts in responding to silica stimulation, we identified that specific SNPs were associated with either single gene expression, or paired gene expression such as CTGF/SPARC and COL1A2/COL3A1, which suggest a strong biological correlation between these genes. Moreover, some SNPs and/or their corresponding genes were found to be associated with both SSc susceptibility and the fibrotic changes of human fibroblast in response to silica stimulation. These SSc susceptibility genes include not only previously identified ones, but also some novel ones, such as HLA-DPB1 and APBA1.These observations supported our original proposal that genetic elements within SSc fibroblasts might contribute to susceptibility to fibrotic process. Integrative studies of genetic and environmental factors with human fibroblasts may facilitate the discovery of potential pathogenesis of SSc. In this year, we will continuously obtain more human fibroblasts, especially SSc fibroblasts. We also will continuously perform stimulation assays of newly obtained human fibroblasts for a better chance to identify genetic components inside SSc patients contributing to susceptibility to environmental stimuli. Meanwhile, we will try to explore which specific bio-pathways associated with which specific genetic factors involved in silica induced fibrotic changes. Therefore, our studies are fulfilled with original proposal in the grant.

15. SUBJECT TERMS
Scleroderma (SSc), fibroblasts, fibrosis, silica, environmental particles, susceptibility.

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Introduction:

This project aims to study interactions between genetic and environmental factors in a viable system - human fibroblasts. Fibroblasts with a scleroderma (SSc) susceptible genetic background may be more vulnerable to environmental triggers. Studies of fibroblasts with and without SSc susceptible backgrounds in response to potential environmental triggers will provide a great opportunity to understand etiopathogenesis of SSc.

Body:

According to our SOW, there are four major tasks that will be performed for this project. 1. We will culture and genotype human fibroblasts obtained from skin biopsies. 2. We will stimulate cultured fibroblasts with silica, cellulose, titanium oxide and carbon particles individually, and then examine potential alterations of fibroblasts in terms of proliferation, ROS production, collagen synthesis, cytokine expression and release. 3. We will apply comprehensive statistical analysis to identify the association of genetic susceptibility genes and these environmental factors in terms of the degree of their profibrotic effects. 4. We will also study specific biological pathways triggered by risk elements in cultured fibroblasts.

During this second year of the project, we have been continuously obtaining skin biopsies from SSc patients and normal controls. Currently, we have a total of 166 skin fibroblast strains including 65 from SSc patients and 101 from normal controls. These primary fibroblast cultures were established from each skin biopsy, and maintained in healthy condition for proposed functional studies.

We have performed stimulation assays on 82 primary fibroblast strains for part of our proposed studies. Each of these 82 fibroblast strain was stimulated with silica particles for evaluation of their responses toward potentially fibrotic changes over 5 different time-points. Meanwhile, we have genotyped 79 of these fibroblast strains with both the HLA subtyping and the genome-wide single nucleotide polymorphism (SNP) profiling (Illumina Human 317 K SNP panel). It should be noted that the studies of performing genome-wide SNP profiling were funded by our other research projects. This addition of genotyping appeared to be very important in understanding complexity of human genetics, especially in association with environmental factors. We examined gene expression changes of COL1A2, COL3A1, CTGF, SPARC and TIMP3 of these fibroblasts in five different time points (0, 1, 2, 3, and 5 days) in response to silica stimulation. The COL1A2, COL3A1, CTGF, SPARC and TIMP3 are three major components of the extracellular matrix (ECM), which are actively involved in fibrotic changes of the tissues. Using longitudinal linear models in the analysis of association between specific genotypes and dynamic changes of gene expression of the fibroblasts in responding to silica stimulation, we identified that specific SNPs were associated with either single gene expression, or paired gene expression (Table 1). Notably, the paired gene expressions of CTGF/SPARC and COL1A2/COL3A1 were associated with multiple SNPs, which suggest a strong biological correlation between these genes. HLA-DQB1*0301 is a positive marker for SSc susceptibility. Interestingly, HLA-DQB1*0301 positive fibroblast strains showed a significant up-regulation (>2-fold increase of transcript) of COL1A2, COL3A1, CTGF and SPARC in response to silica stimulation after 24 hours, which was significantly different from the response of HLA-DQB1*0301 negative fibroblast strains ($P < 0.05$ for all 4 genes).

While HLA-DQB1*0301 is a previously identified SSc genetic marker, we are recently exploring novel SSc susceptibility markers using a genome-wide SNP association (GWAS) approach. Collaborating with Dr. Eun Bong Lee (Seoul National University College of Medicine, Seoul, Korea), we demonstrated that specific SNPs of the HLA-DPB1 and -DPB2 were strongly associated with SSc susceptibility in both Koreans and US Caucasians (see attached manuscript 1). Incorporating this information into our data of gene expression changes of the fibroblasts, we revealed that specific SNPs of HLA-DPB1 also was associated with gene expression changes of the COL1A2 of the fibroblasts in response to silica stimulation ($p = 0.00042$). Moreover, several other potential SSc susceptibility regions identified from the Dr. Lee's GWAS also showed an overlap with the genetic regions associated with gene expression changes of the ECM components of the fibroblasts in responds to silica stimulation. The figure 1 demonstrates an example of the association between genetic regions and expression changes of the COL1A2 of the fibroblasts in response to silica stimulation. The figure 2 represents a GWAS results from Dr. Lee's data that shows log P-values of the genome-wide SNPs in association with SSc. Comparing both figures, we can find the overlapped chromosomal regions such as on chromosome 1, 3, 6 and 10. In addition to Dr. Lee's GWAS studies of SSc, Dr. Maureen Mayes, a professor in our division of Rheumatology in UT Health Science Center, just completed the GWAS studies of SSc with 1678 SSc Caucasian SSc patients and 5530 Caucasian controls (Dr. Mayes' personal communication). We had a chance to pre-examine this unpublished GWAS data. Remarkably, multiple SNPs that showed an association with SSc susceptibility in the GWAS studies were overlapped with the SNPs that also were associated with the gene expression of the ECM of the fibroblasts in response to silica stimulation (Table 2). Further studies of the genes corresponding to these SNPs may provide important knowledge to understand pathogenesis of SSc. For instance, the ABCA7 (ATP-binding cassette, subfamily A, member 7) encodes a transmembrane protein involved in energy-dependent transport of a wide spectrum of substrates. Dysregulation of ATP-dependent membrane transportation may lead to various pathological events associated with vascular and immune functions. The APBA1 (amyloid beta A4 precursor protein-binding, family A, member 1) is a candidate gene for Alzheimer disease. However, serum amyloid protein was reported to be lower in SSc patients (*Pilling D., et al. J Immunol. 2003;171:5537-46*). According to Philing's studies, low levels of SAP may augment pathological processes leading to fibrosis. The EGKD (diacylglycerol kinase delta) is a gene encoding protein regulates protein kinase C and epidermal growth factor receptor signaling *(Proc. Nat. Acad. Sci.* 103: 15485-15490, 2006*).* The RBMS3 (RNA-binding motif protein, single strand-interacting 3) is a gene closely related to c-myc involving in regulation of DNA replication, gene transcription, apoptosis and cell cycle.

Overall, above data strongly support our original proposal that genetic elements within SSc fibroblasts might contribute to susceptibility to fibrotic process. Integrative studies of genetic and environmental factors with human fibroblasts may facilitate the discovery of potential pathogenesis of SSc.

Our efforts in studying specific biological pathways triggered by risk elements in cultured fibroblasts have been continued through last year. Previously, we demonstrated that silica stimulation on fibroblasts induced TGF-β signaling (reported in original proposal), which may contribute to fibrosis. Recently, we identified that SPARC inhibition attenuated profibrotic effects of TGF-β on human fibroblasts. Last year, we applied SPARC siRNA in bleomycin-induced mouse fibrotic model, and demonstrated an anti-fibrotic effect of SPARC inhibition *in vivo* (attached manuscript 2). Although, the *in vivo* studies of anti-fibrosis were funded by other projects, the results represent a successful example of translational studies of medical diseases.

Table 1. Associations between SNPs and gene expression of COL1A2, COL3A1, CTGF, SPARC and TIMP3 of human fibroblasts in response to silica stimulation

			P-value for testing genetic effect to gene expression				
Associated genes	rs number	Gene Name	COL1A2	COL3A1	CTGF	SPARC	TIMP3
COL1A2/COL3A1	rs10484710	COL21A1	6×10^{-13}	1.7×10^{-5}	> 0.01	> 0.01	> 0.01
COL1A2/COL3A1	rs5972761	DMD	7.4×10^{-12}	8.8×10^{-4}	> 0.01	> 0.01	> 0.01
COL1A2/COL3A1	rs5972763	DMD	7.4×10^{-12}	8.8×10^{-4}	> 0.01	> 0.01	> 0.01
COL1A2/COL3A1	rs3734061	FAT2	5.4×10^{-12}	7.9×10^{-4}	> 0.01	> 0.01	> 0.01
COL1A2/COL3A1	rs998076	FAT2	5.4×10^{-6}	3×10^{-4}	> 0.01	> 0.01	> 0.01
COL1A2/COL3A1	rs2288777	FAT2	3.7×10^{-4}	1.3×10^{-3}	> 0.01	> 0.01	> 0.01
COL1A2/COL3A1	rs1410871	GBP4	7.1×10^{-8}	6.5×10^{-4}	> 0.01	> 0.01	> 0.01
COL1A2/COL3A1	rs2196426	hCG_2015138	7.2×10^{-14}	1×10^{-7}	> 0.01	> 0.01	> 0.01
COL1A2/COL3A1	rs4803831	OPA3	4.2×10^{-8}	1.3×10^{-5}	> 0.01	> 0.01	> 0.01
COL1A2/COL3A1	rs3809251	PARP11	7.9×10^{-3}	1.3×10^{-7}	> 0.01	> 0.01	> 0.01
COL1A2/COL3A1	rs2061783	XRCC4	2.9×10^{-14}	6.3×10^{-8}	> 0.01	> 0.01	> 0.01
COL1A2/COL3A1	rs10947541	DEF6	2.9×10^{-14}	6.3×10^{-8}	> 0.01	> 0.01	> 0.01
COL1A2/COL3A1/SPARC	rs2289722	C4orf20	2.1×10^{-5}	3.6×10^{-12}	> 0.01	7.5×10^{-5}	> 0.01
CTGF/SPARC	rs6810854	ACOX3	> 0.01	> 0.01	3.4×10^{-15}	8.8×10^{-6}	> 0.01
CTGF/SPARC	rs9375513	C6orf174	> 0.01	> 0.01	1.4×10^{-8}	1.4×10^{-3}	> 0.01
CTGF/SPARC	rs2236026	KIAA0408	> 0.01	> 0.01	1.4×10^{-8}	1.4×10^{-3}	> 0.01
CTGF/SPARC	rs3922703	PLCXD2	> 0.01	> 0.01	1.5×10^{-8}	9.4×10^{-5}	> 0.01
CTGF/COL3A1	rs3788926	ZRSR2	> 0.01	1.1×10^{-8}	2.8×10^{-5}	> 0.01	> 0.01
CTGF/TIMP3	rs11702035	C21orf51	> 0.01	> 0.01	1.4×10^{-7}	> 0.01	8.8×10^{-5}
CTGF/SPARC/TIMP3	rs10507399	HSPH1	> 0.01	> 0.01	7.8×10^{-9}	2.9×10^{-6}	3.9×10^{-8}
SPARC/CTGF/COL1A2/COL3A1	rs13082485	PIK3CA	2.5×10^{-7}	1.8×10^{-3}	2.5×10^{-3}	1.9×10^{-7}	> 0.01
COL1A2	rs437444	CELSR2	1.1×10^{-7}	> 0.01	> 0.01	> 0.01	> 0.01
COL1A2	rs10478113	MCC	1×10^{-7}	> 0.01	> 0.01	> 0.01	> 0.01
COL1A2	rs3793726	PRKCQ	1.5×10^{-7}	> 0.01	> 0.01	> 0.01	> 0.01
COL1A2	rs7918923	PRKCQ	1.6×10^{-7}	> 0.01	> 0.01	> 0.01	> 0.01
COL1A2	rs6445245	PTPRG	1.5×10^{-7}	> 0.01	> 0.01	> 0.01	> 0.01
COL1A2	rs2864780	RAB31	7.8×10^{-8}	> 0.01	> 0.01	> 0.01	> 0.01
COL1A2	rs3731895	RESP18	6×10^{-8}	> 0.01	> 0.01	> 0.01	> 0.01
COL3A1	rs11564394	CDH2	> 0.01	1.5×10^{-7}	> 0.01	> 0.01	> 0.01
COL3A1	rs11754507	DSE	> 0.01	1.6×10^{-11}	> 0.01	> 0.01	> 0.01
COL3A1	rs7052934	SPANXN3	> 0.01	3.4×10^{-8}	> 0.01	> 0.01	> 0.01
COL3A1	rs11817964	ZNF365	> 0.01	1.6×10^{-7}	> 0.01	> 0.01	> 0.01
TIMP3	rs10499156	LAMA2	> 0.01	> 0.01	> 0.01	> 0.01	5.4×10^{-8}
TIMP3	rs13154825	SLIT3	> 0.01	> 0.01	> 0.01	> 0.01	8.1×10^{-9}
TIMP3	rs1476707	C19orf29	> 0.01	> 0.01	> 0.01	> 0.01	6.1×10^{-9}

Table 2. The SNPs associated with both SSc susceptibility and gene expression changes of the fibroblasts in response to silica stimulation.

SNP ID	Gene symbo	Gene ID	P-value for SNP-SSc association	P-value for testing genetic effect on gene expression	Associated gene expression of
rs3752228	ABCA7	NM_019112.2	0.0004622	0.0046	COL1A2
rs1831555	APBA1	NM_001163.2	0.0001345	0.0077	CTGF
rs1977552	APBA1	NM_001163.2	0.0001478	0.0068	CTGF
rs2781530	APBA1	NM_001163.2	0.0002292	0.0077	CTGF
rs673576	C21orf88	NM_153754.1	0.0002405	0.0057	TIMP3
rs7584554	DGKD	NM_152879.2	0.0002883	0.0017	CTGF
rs4981200	NPAS3	NM_022123.1	0.0006308	0.0067	CTGF
rs4981200	NPAS3	NM_022123.1	0.0006308	0.0049	SPARC
rs4981200	NPAS3	NM_022123.1	0.0006308	0.0068	COL3A2
rs3773046	RBMS3	NM_001003792	0.0001188	0.0072	COL1A2

Figure 1. Identification of genetic loci associated with high sensitivity of fibroblasts over-expressing the *COL1A2* in response to silica stimulation. (X axis indicates SNP positions on different chromosomes, Y axis indicates log p values of association between over-expression of the COL1A2 and SNP genotypes). The gene expression of the COL1A2 was examined with real-time RT-PCR. The genome-wide SNP typing on human fibroblasts was performed with Illumina's human 317 K SNP panel.

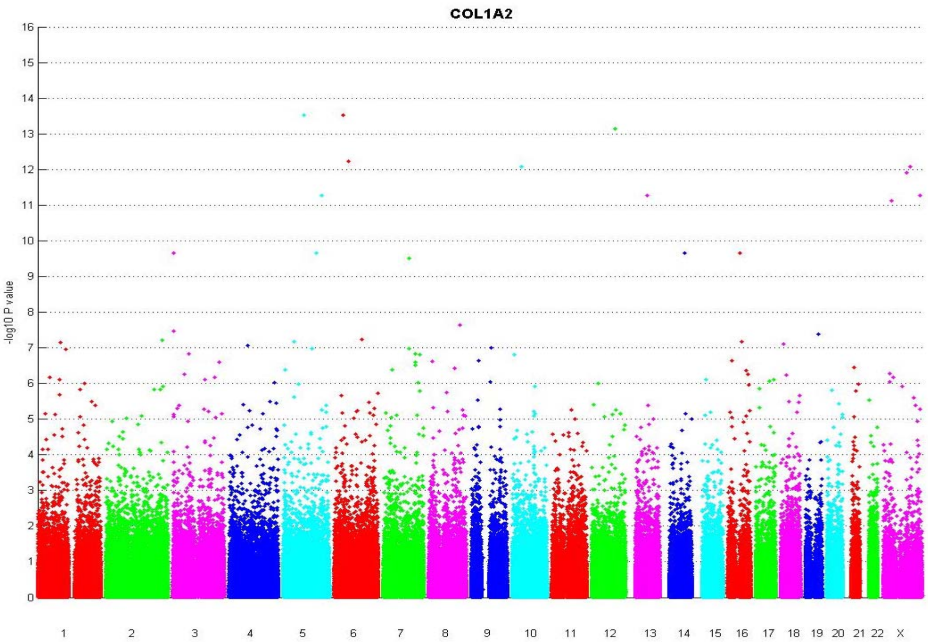
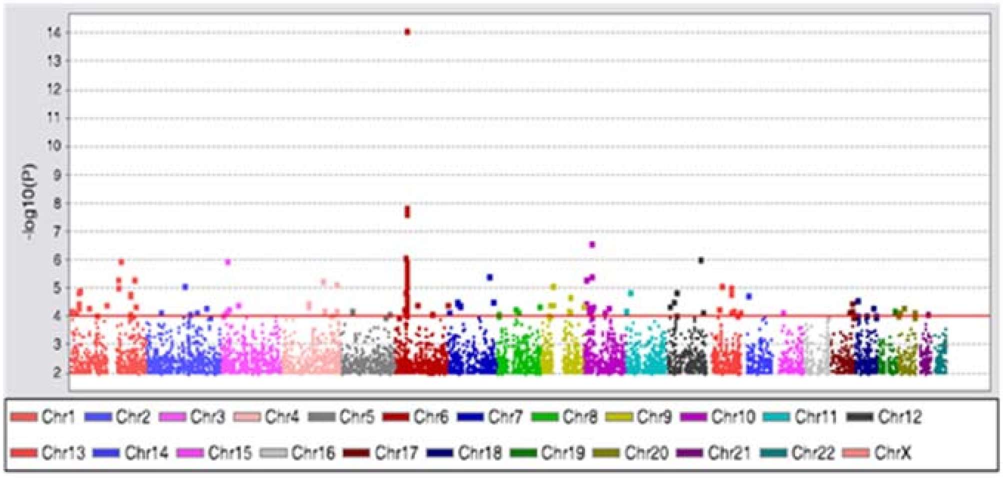


Figure 2. Identification of the major loci associated with systemic sclerosis in genome wide scan. A total of 440,734 SNPs were evaluated in 133 patients with SSc and 557 healthy controls. Distribution of $-\mathrm{Log}_{10}$ P values are plotted against chromosomes. The genome-wide SNP typing was performed with Affymetrix Whole Genome Human SNP Array 5.0.



Key Research Accomplishments

- Obtained a total of 166 human fibroblast strains (65 SSc patients and 101 normal controls).
- Completed silica stimulation in 82 fibroblast strains and obtained RNA and protein extracts from each of the experiments.
- Completed genotyping for 79 fibroblast strains (34 SSc and 45 controls) with both HLA subtyping and genome-wide SNP profiling.

- Identified that the paired gene expressions of CTGF/SPARC and COL1A2/COL3A1 were associated with multiple SNPs, which suggest a strong biological correlation between these genes.
- Identified some novel genes and SNPs associated with both SSc susceptibility and human fibroblast response to silica stimulation in terms of their gene expression changes of the ECM components.
- Demonstrated that specific inhibition of SPARC, a major ECM component, attenuated mouse fibrosis induced by bleomycin *in vivo*.

Reportable Outcomes

During this second year period, we reported that SPARC inhibition attenuated profibrotic effects of TGF-β *in vitro* and bleomycin *in vivo* (oral presentation) in annual meeting of American College of Rheumatology 2008. We have one full article accepted by the European Journal of Human Genetics (2009). We have two manuscripts submitted for publication (one received comments for revision) and two manuscripts in preparation.

Importantly, we established a total of 166 human fibroblast strains (65 SSc and 101 controls), which is close to our minimum target of 200 (100 SSc and 100 controls) that can be used for many studies toward human health.

Abstract:

1. Wang J, Lai S, Sonnylal S, Arnett F.C., Crombrugghe B and **Zhou XD**. Application of Sparc and Ctgf siRNAs in fibrotic murine models of scleroderma (SSc) *in vitro* and *in vivo.* Arthritis & Rheuma 2008;58 (9); supplement for ACR annual meeting.

2. **Zhou XD**, Tan FK, Guo XJ, Tejpal N, Kahan BD, Stepkowski SN, Arnett FC. Up-take Of Silica And Carbon Nanotubes By Human Macrophages Induces Activation Of T Cells And Fibroblasts *In Vitro* - Potential Implication For Pathogenesis Of Fibrosing Diseases. Arthritis & Rheuma 2007;56 (9);s245. supplement for ACR annual meeting.

Referred articles (underline for corresponding author):

1. Xiong M, Arnett FC, Xiong H and **Zhou XD**. Differential Dynamic Properties of Scleroderma Fibroblasts in Response to Perturbation of Environmental Stimuli -Application of State-Space Model in Studies of human Complex Disease. PLoS ONE, 2008 Feb, 3(2):e1693.
2. Momiao Xiong, Gang Peng , Li Luo , Yun Zhu , Pengfei Hu , Shengjun Hong , Jinying Zhao , **Zhou XD**, John Reveille, Li Jin , Christopher Amos. Gene and Pathway-Based Analysis -Second Wave of Genome-wide Association Studies. European Journal of Human Genetics (2009) (in press).

Manuscript submitted (see attachment)

1. **Zhou XD**, Lee JU, Arnett FC, Xiong MM, Park MY, Yoo YK, Shin ES, Reveille JD, Mayes MD, Kim JH, Song R, Choi JY, Park JA, Lee YJ, Lee EU, Song YW, Lee EB. HLA-DPB1 and DPB2 are culprit genetic loci for susceptibility to systemic sclerosis, Genome-wide association study in Koreans with Replication in North Americans. Arthritis Rheum (2009). (received comments for revision)
2. Wang JC, Lai SL, Guo XJ, Zheng XF, de Crembrugghe B, Arnett FC and **Zhou XD**. Attenuation of fibrosis in vitro and in vivo with Sparc siRNA. (submitted to Arthritis Rheum 2009)

Manuscript in preparation

1. **Zhou XD**, Jagannath C, Tan FK, Guo XJ, Tejpal N, Kahan BD, Stepkowski SN, Arnett FC. Up-take of silica and carbon nanotubes by human macrophages induces activation of T cells and fibroblasts in vitro – potential implication for pathogenesis of fibrotic diseases.

2. JC Wang, Xiong H, Tan FK, Arnett FC, **Zhou XD**. Studies of genetic and environmental factors in human fibroblasts showed novel gene-gene interactions.

Conclusion

During this second year of the project, we established multiple primary cell strains from normal controls and SSc patients. We performed stimulation assays with silica in 82 primary fibroblast strains. Our results showed that silica activate fibroblasts toward fibrotic changes. However, different fibroblast strains obtained from different individuals showed different responses in terms of the gene expression of the ECM components. Using longitudinal linear models in analysis of association between specific genotypes and dynamic changes of gene expression of the fibroblasts in responding to silica stimulation, we identified that specific SNPs were associated with either single gene expression, or paired gene expression such as CTGF/SPARC and COL1A2/COL3A1, which suggest a strong biological correlation between these genes. Moreover, some SNPs and/or their corresponding genes were found to be associated with both SSc susceptibility and the fibrotic changes of human fibroblast in response to silica stimulation. These SSc susceptibility genes include not only previously identified ones, but also some novel ones, such as HLA-DPB1 and APBA1.These observations supported our original proposal that genetic elements within SSc fibroblasts might contribute to susceptibility to fibrotic process. Integrative studies of genetic and environmental factors with human fibroblasts may facilitate the discovery of potential pathogenesis of SSc. In this year, we will continuously obtain more human fibroblasts, especially SSc fibroblasts. We also will continuously perform stimulation assays of newly obtained human fibroblasts for a better chance to identify genetic components inside SSc patients contributing to susceptibility to environmental stimuli. Meanwhile, we will try to explore which specific bio-pathways associated with which specific genetic factors involved in silica induced fibrotic changes. Therefore, our studies are fulfilled with original proposal in the grant.

Appendices (two manuscripts submitted for publication)

HLA-DPB1 and DPB2 are culprit genetic loci for susceptibility to systemic sclerosis

-Genome-wide association study in Koreans with Replication in North Americans

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Abstract

We performed a genome-wide association study using the Affymetrix Human SNP Array 5.0 in 137 patients with systemic sclerosis and 564 controls from Korea. The SNPs (rs3128930, rs7763822, rs7764491, rs3117230 and rs3128965) of HLA-DPB1 and –DPB2 on chromosome 6 formed a distinctive peak with log p-values ($p < 1 \times 10^{-14}$) for association with SSc susceptibility. Subtyping analysis of HLA-DPB1 showed that DPB1*1301 ($p = 7.61 \times 10^{-8}$) and DPB1*0901 ($p = 2.56 \times 10^{-5}$) are the most susceptible subtypes for SSc in Koreans. We then replicated the results in 1,106 SSc patients and 2,747 controls from a US Caucasian population. Remarkably, two pairs of SNPs, rs7763822/rs7764491 and rs3117230/rs3128965, showed strong association with Caucasian SSc patients who have either circulating anti-DNA topoisomerase I or anti-centromere autoantibodies, respectively. Therefore, our GWAS and confirmatory studies revealed that the region of HLA-*DPB1* and –*DPB2* contains the most susceptible loci to SSc, especially those patients with anti-topoisomerase I or anti-centromere autoantibodies.

Introduction

Systemic sclerosis (SSc) is a rare and complex connective tissue disease of unknown etiology characterized by fibrosis and vasculopathy of skin and internal organs, as well as several, mutually exclusive, disease- specific circulating autoantibodies[1]. SSc can be clinically sub-classified based on patterns of skin fibrosis into limited and diffuse forms. In addition, the majority of SSc patients (90%) have circulating anti-nuclear autoantibodies (ANA)[2]. The three most common autoantibodies (auto-Abs) are anti-DNA topoisomerase I (topo I), anti-RNA polymerase III, and anti-centromere antibodies, in which the first two auto-Abs tend to be associated with diffuse SSc[2; 3], the last one being strongly correlated with limited SSc, although these associations are not complete[2; 4].

Genetic predisposition is widely believed to contribute to SSc. Several genetic regions have been reported to be associated with SSc susceptibility, including the major histocompatibility complex (MHC) or HLA-class II[5], protein tyrosine phosphatase non-receptor 22 (*PTPN22)*[6], allograft inflammatory factor 1 (AIF1)[7], tumor necrosis factor (TNF)[8], cytotoxic lymphocyte antigen-4 (CTLA-4)[9], transforming growth factor (TGF-β)[10] and connective tissue growth factor (CTGF)[11]. However, the low prevalence of SSc (approximately 0.00070--0.049%)[12; 13] and clinical/serological heterogeneity make genetic studies of SSc difficult with some differing results reported for the same genes in different ethnic groups. To address such difficulties, we conducted a two-step genetic association study in four independent populations to identify the susceptibility markers for SSc.

Material and Methods

Study Subjects

We examined 4 different ethnic populations (Koreans, Caucasian Americans, African Americans and Hispanics). Korean study population was composed of 151 SSc patients diagnosed according to the ACR preliminary criteria for SSc[14]. All the patients were enrolled from Seoul National University Hospital between January 1998 and 2007. Genomic DNA was extracted from whole blood using standard methods. A total of 137 cases which passed the DNA quality check were entered into the GWAS using Affymetrix Genome-Wide Human SNP Array 5.0. A total of 133 cases which showed >95% of call rates, were finally entered into the case-control analysis. The mean age at diagnosis was 42 years ranging from 4 to 67 years. Mean duration of the disease was 10 years and the mean time from diagnosis to blood sampling was 5 years.

The 600 healthy controls were randomly selected from 10,000 healthy Koreans belonging to Korean Association Resource (KARE) Project, based on the frequency-matching on sex with the cases. The mean age of the controls was 52.5 years. The same platform (Affymetrix Genome-Wide Human SNP Array 5.0) was used for the whole-genome scan of the controls. After excluding cases with low call rate less than 95%, mismatched sex and potential relatives, a total of 557 controls were finally entered into the case-control analysis. The institutional review board of Seoul National University Hospital approved the study and all patients and controls provided written consents.

SSc patients of Caucasians, African Americans and Hispanics who met the ACR criteria for SSc and corresponding controls (sex and ethnically matched) were enrolled in the Division of Rheumatology, University of Texas Health Science Center at Houston (UTHSC-Houston)[6; 7]. In addition, 2,300 Caucasian controls were selected from NIH data base of Genotype and Phenotype (dbGaP) found at http://www.ncbi.nlm.nih.gov/gap. The study was approved by the institutional review boards of the University of Texas Health Science Center at Houston. All the individual patients and controls provided written consent.

GWAS analysis

Among the 500,568 SNPs present in Affymetrix Whole Genome Human SNP Array 5.0, 440,734 SNPs were accessible after excluding hidden SNPs. Q-Q plot was obtained in the condition of p-value > 0.0001 for Hardy-Weinberg equilibrium and call-rate > 0.95 (Figure 1). Finally cluster quality analysis was performed, which led to 349,209 SNPs which can be analyzed. The most significant SNPs were determined to be rs3128930, re7763822, rs7764491, rs3117230 and rs3128965 and they went into the fine mapping process.

Fine mapping

For Koreans, we performed a fine mapping focusing on HLA-*DPB1* and *-DPB2* regions with 137 SSc cases and 548 healthy controls in whom DNA was available. For HLA-DPB1, highly variable exon 2 of the gene was DNA-sequenced to determine subtype of HLA-DPB1. For the other region including

HLA-DPB2, total 22 tag SNPs were selected with r^2 threshold of 0.8 and minor allele frequency over 5% in HapMap Japanese panel data (release 22) using the Haploview version 4.1[15; 16]. In tag SNPs, 17 SNPs which are included in Affymetrix SNP chip were forced to be included.

For replication studies, TaqMan Assays were used for SNPs rs3128930, re7763822, rs7764491, rs3117230 and rs3128965 genotyping with an ABI 7900HT Fast Real-Time PCR System in Caucasian, African American and Hispanic populations. Genotyping results of all five SNPs passed quality tests for Hardy-Weinberg ($p > 0.001$) and calling rate (>95%). Two groups of Caucasian controls from the NIH data base and the UTHSC-Houston showed concordant association with Caucasian SSc patients.

Statistical analysis

The association of specific SNPs with the disease or a subset of the disease was analyzed by the comparison of minor allele frequencies of the cases and controls, with significance determined by p-values of chi-square tests, Cochran-Armitage Trend test, and Jonckheere-Terpstra tests. The odds ratio of cases' having a selected SNP compared with the controls' and its relevant 95% confidence intervals were also determined. For GWAS, The threshold for declaring significance after Bonferroni correction for adjusting multiple tests was $p<1.43\times10^{-7}$. It was $p<2.2\times10^{-3}$ for the fine mapping study (n=22) and $p<0.01$ for the replication study (n=5). All the association tests were based on the comparison of alleles. PLINK[17] and SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) were used for the statistical analysis. Linkage disequilibrium analysis for HLA-DPB1 and –DPB2 regions was performed with Haploview, version 4.1[16].

Results

We first examined a Korean population who are relatively homogenous in which 62.2% of patients are positive for anti-topo I auto-Abs (based on Korean patients enrolled in this project). We performed a genome-wide association study (GWAS) in 137 Korean SSc patients and 564 sex-matched Korean controls using the Affymetrix Human SNP Array 5.0 containing 440,734 accessible human SNPs. The GWAS showed a distinctive peak of SNP log p-value ($p = 7.84 \times 10^{-15}$ for association with SSc) (Figure 1). The peak was formed with the SNPs rs3128930, rs7763822, rs7764491, rs3117230 and rs3128965, which were located in the region of HLA-DPB1 and –DPB2 (a psuedogene) on chromosome 6p (Figure 1, 2). Fine mapping analysis of this region confirmed that rs3128930, rs7763822 and rs7764491 were the culprit SNPs for associations with Korean SSc (Figure 2, Table 1). Interestingly, the association was even stronger in patients who were positive for anti-topo I auto-Abs (rs3128930, $p = 1.70 \times 10^{-22}$, OR 5.15, 95% CI 3.62-7.34) (Table 1). These SNPs also were associated with the diffuse form of SSc, but not the limited forms of SSc (Supplementary Table 1). Subtyping of HLA-DPB1 showed that HLA-DPB1*1301 (21.0% in SSc vs. 5.5% in controls), DPB1*0901 (12.0% vs. 2.6%) and DPB1*030101 (10.0% vs. 4.3%) were significantly more represented in anti-topo 1 positive patients than controls (Table 2). SNPs corresponding to previous SSc associated reported genes, such as *PTPN22*, *AIF1*, *TNF*, *CTLA-4*, *TGFB* and *CTGF*, fell within the significance thresholds of 10^{-5}-10^{-6} advocated for gene-based scans, as well as the Bonferroni correction for multiple comparisons[18].

To confirm these results, we used TaqMan Assays to reexamine the 5 SNPs with the strongest association from the Korean GWAS screen in 1,106 US Caucasian SSc patients (collected from US), of whom 16% were positive for anti-topo I, and 2,747 normal controls, of which 447 were from our local collections (Houston, Texas, US.) and 2,300 were from the NIH data base of Genotype and Phenotype (dbGaP) (http://www.ncbi.nlm.nih.gov/gap). The SNPs rs7763822 and rs7764491 showed highly significant associations with SSc patients who were positive for anti-topo I auto-Abs ($p = 7.58 \times 10^{-17}$ and 4.84×10^{-16}, respectively) (Table 3). The HLA-DPB1*1301 allele which occurs in only 3% of US Caucasians was found in 25% of anti-topo I positive patients and conferred the strongest risk by exact logistic regression (p=0.0001, OR=14) of any HLA class II allele (unpublished results). The SNPs rs3128965 and rs3117230 showed strong associations with SSc patients who were positive for anti-centromere auto-Abs ($p = 3.20 \times 10^{-5}$ and 1.12×10^{-3}, respectively) (Table 3). In addition, the pair of anti-topo I associated SNPs also showed a weaker association with the diffuse form of SSc (p = 0.0070 and 0.014 for rs7763822 and rs7764491 respectively) (Supplementary Table 2). The genetic concordance of the patients with anti-topo I positivity and the diffuse form of SSc supports clinical observations that these two traits within SSc commonly overlap[2; 3]. However, the anti-centromere associated SNP pairs did not show strong associations with the limited form of SSc that usually occurs in patients with anti-centromere auto-Abs[2; 4]. The SNP rs3128930 showed only a marginal p value of 0.041 in the limited form of SSc patients who were positive for anti-centromere auto-Abs (Supplementary table 2). Further analysis of Caucasian patients who are negative for anti-topo I or anti-centromere autoantibodies indicated that they have marginal or no association with the genotypes of all the 5 SNPs (Table3). In contrast, highly significant differences were observed in the comparisons of patients with and without anti-topo I or anti-centromere autoantibodies using corresponding anti-topo I or anti-centromere associated SNP pairs (rs7763822 and rs7764491 pair, $p \le 2.86 \times 10^{-18}$ or rs3128965 and rs3117230 pair, 4.77×10^{-4} respectively) (Supplementary Table 3). Interestingly, marginally significant differences ($0.05 > p \ge 0.01$) were also observed in the comparison of patients with the limited and the diffuse forms of SSc using both pairs of SNPs but the autoantibody associations were the strongest, perhaps because HLA alleles are specific immune response genes (Table 3, and supplementary Table 2).

Finally, we studied these same SNPs in African Americans and Hispanics with limited numbers of cases and controls (70 cases vs. 90 controls and 61 cases vs. 90 controls, respectively). Although, the numbers of subjects examined in these two populations were small, the SNPs rs7764491 and rs7763822 showed a consistently strong association with anti-topo I positive SSc in both African Americans ($p=9.05 \times 10^{-3}$, OR=4.23, 95% CI=1.33-13.48 for both SNPs) and Hispanics (7.98×10^{-4}, OR=5.51, 95% CI=1.85-16.43 and $p=7.21 \times 10^{-4}$, OR=5.57, 95% CI=1.87-16.62, respectively) (Supplementary Table 4, Supplementary Table 5).

Discussion

For human complex diseases, such as SSc and systemic lupus erythematosus (SLE), there have been inconsistent reports of genetic associations from different study populations (e.g. *CTGF*[11; 19]*, PTPN22*[6; 20-22] *and, TGF-beta*[10; 23; 24]). Our findings of SSc-associated HLA-DPB1 and -DPB2 on the basis of autoantibodies, represent the first replicable report in Asians, Caucasians, African-Americans and Hispanics. These complementary studies in four independent populations provide strong support for these identified genetic makers to confer susceptibility to subgroups of SSc patients. HLA-DPB1, located centromeric to other HLA class II molecules, shows relatively low linkage disequilibrium with other extended MHC haplotypes[25]. Although it was not studied as extensively as HLA-A, -B, -C or -DR, it has similar antigen-presentation function to activate CD4+ T cells[26]. Its genetic polymorphisms have been found to be associated with chronic berylliosis[27], graft-vs-host disease[28], juvenile rheumatoid arthrtitis[29], insulin dependent diabetes mellitus[30] and sarcoidosis[31]. Some studies have suggested the possible roles of HLA-DPB1 in SSc, in the context of HLA-A, B, C and DR molecules[32-34]. However, the results of our study suggest that HLA-DPB1 may be the most important susceptible gene to SSc, especially to those patients with auto-antibodies to topo-1.

Our studies indicate that sub-classification and population origin may be two critical factors in identifying genetic susceptibility markers for SSc. Such is evidenced in our lack of association in Caucasian SSc with any of the 5 SNPs found in the Korean GWAS until we took into account those subgroups defined by clinically diffuse and limited forms, but especially by specific autoantibody status (anti-topo I and anti-centromere positives). In addition, this notion may also explain previous inconsistent reports of SSc associated genes in different populations, as well as the fact that these genes were not identified in our GWAS of Koreans.

Importantly, the genetic differentiations of SSc with distinctive serological and clinical features in our studies suggest that SSc should not be considered as a single disease. While SSc, like other human complex diseases, is currently incurable, sub-classification of SSc on the basis of genetic polymorphisms for disease susceptibility may provide a new dimension for exploring pathogenesis and treatment of this disease. Our results strongly indicate that sub-classification of SSc on the basis of autoantibodies against topo I and centromeric proteins is important in defining disease susceptibility genes in this heterogeneous disease.

Keywords: Systemic sclerosis, Genome wide association study, HLA-DPB1, Anti-topoisomerase I antibody

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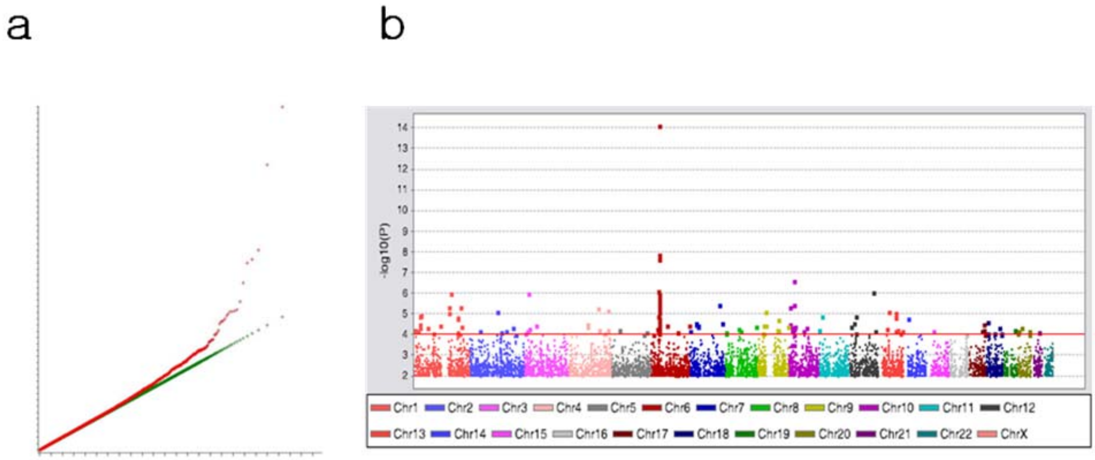
Figures legends

Figure 1. Identification of the major locus associated with SSc in genome wide scan of Koreans. A total of 440,734 SNPs were evaluated in 133 patients with SSc and 557 healthy controls. A) Quantile-quantile plot compares the distributions of observed P-values with expected null P values. B) Distribution of $-\mathrm{Log}_{10}$ P values are plotted against chromosomes.

Figure 2. HLA-DPB as a culprit region for susceptibility to systemic sclerosis. $-\mathrm{Log}_{10}$P values are depicted around the HLA-DPB region. All genes in the region are also displayed above the linkage disequilibrium (LD) map. LD between pairs of SNPs is depicted with linkage blocks in which bright red block represents disequilibrium coefficient (D') of 1.0 and white block D' of 0.0. Orange bar above the LD map represents high LD SNPs which show significant association (r^2>0.8) with culprit SNPs.

Figure 1


a
b
-log10(P)
14
13
12
11
10
9
8
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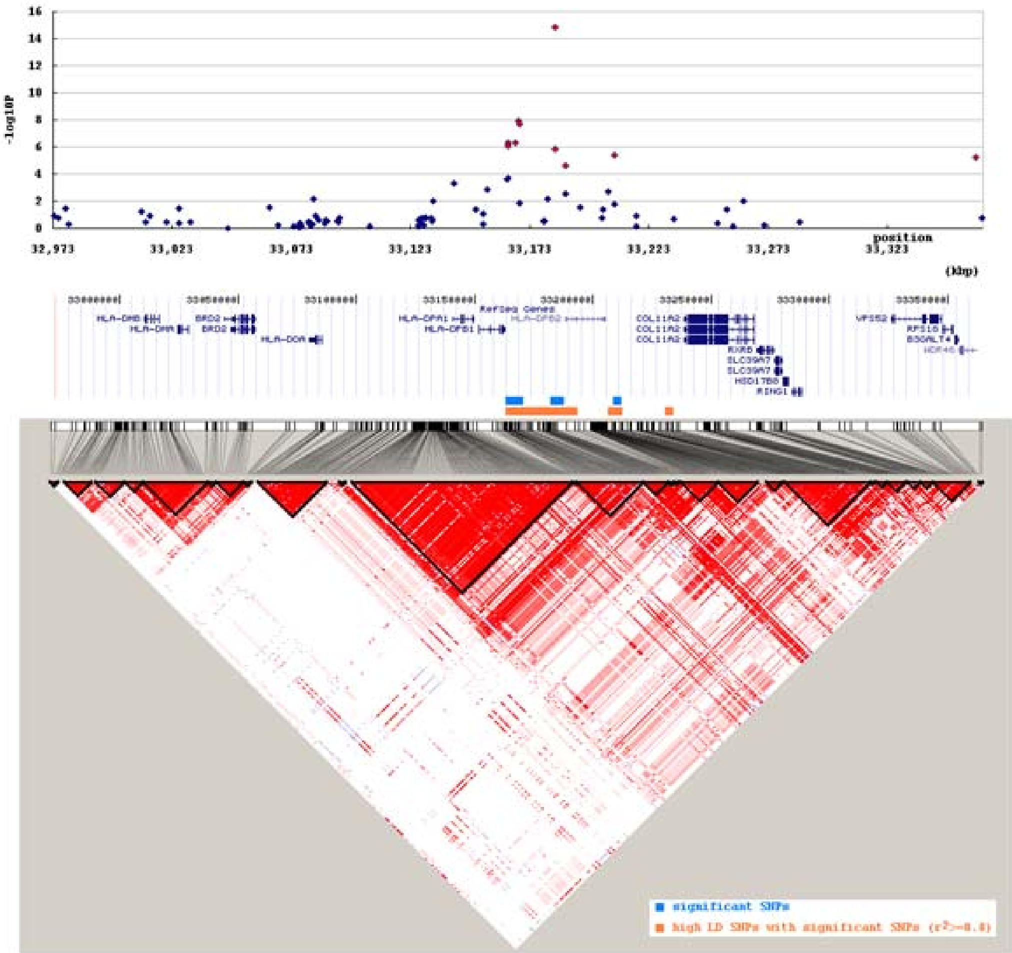
6
5
4
3
2
Chr1 Chr2 Chr3 Chr4 Chr5 Chr6 Chr7 Chr8 Chr9 Chr10 Chr11 Chr12
Chr13 Chr14 Chr15 Chr16 Chr17 Chr18 Chr19 Chr20 Chr21 Chr22 ChrX

Figure 2


-log10P
16
14
12
10
8
6
4
2
0
32,973
33,023
33,073
33,123
33,173
33,223
33,273
33,323
position
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33000000
33050000
33100000
33150000
33200000
33250000
33300000
33350000
RefSeq Genes
HLA-DMB
HLA-DMA
BRD2
BRD2
HLA-DOA
HLA-DPA1
HLA-DPB1
HLA-DPB2
COL11A2
COL11A2
COL11A2
RXRB
SLC39A7
SLC39A7
HSD17B8
RING1
VPS52
RPS18
B3GALT4
significant SNPs
high LD SNPs with significant SNPs (r^2>=0.8)

Table 1. Associaiton of SNPs rs3128930, rs7764491 and rs3128965 with Korean SSc patients with and without autoantibodies to DNA topoisomerase I and centromere protein.

Alleles	Systemic sclerosis					Controls
(Mninor allele)	Anti-topoisomerase I Ab[b]		Anti-centromere Ab		Total	
	(+)	(-)	(+)	(-)		
	(n=79)	(n=48)	(n=16)	(n=117)	(n=133)	(n=557)
rs3128930 (A)						
Frequency	0.47	0.15	0.13	0.39	0.36	0.15
P-value[a]	1.70×10^{-22}	0.93	0.705	2.38×10^{-17}	7.84×10^{-15}	-
OR (95% CI)[a]	5.15(3.62-7.34)	0.97 (0.54-1.76)	0.82(0.28-2.35)	3.63(2.66-4.95)	3.17(2.35-4.28)	-
rs7764491 (C)						
Frequency	0.21	0.073	0.063	0.17	0.16	0.057
P-value[a]	1.18×10^{-11}	0.51	0.886	2.13×10^{-9}	2.13×10^{-8}	-
OR (95% CI)[a]	4.40(2.78-6.98)	1.31(0.58-2.95)	1.11(0.26-4.76)	3.44(2.25-5.26)	3.13(2.06-4.74)	-
rs3128965 (A)						
Frequency	0.26	0.073	0.063	0.21	0.19	0.093
P-value[a]	7.78×10^{-10}	0.506	0.553	1.44×10^{-7}	2.29×10^{-6}	-
OR(95% CI)[a]	3.40(2.26-5.12)	0.76 (0.34-1.69)	0.65(0.15-2.75)	2.64(1.82-3.83)	2.36(1.64-3.40)	-

a.Each subset versus controls b.Missing information of anti-topoisomerase I antibody status in 6 patients; Ab, antibody; CI, confidence interval

Table 2. Association of HLA-DPB1 allelic subtypes with systemic sclerosis in Koreans.

HLA alleles	Systemic sclerosis					Controls
	Anti-topoisomerase-I Ab[b]		Anti-centromere Ab		Total	
	(+)	(-)	(+)	(-)		
	(n=82)	(n=49)	(n=16)	(n=121)	(n=137)	(n=548)
***1301**						
Frequency	0.21	0.05	0.031	0.17	0.15	0.055
P-value[a]	4.05×10^{-12}	0.876	0.562	3.25×10^{-9}	7.61×10^{-8}	-
OR (95% CI)[a]	4.52(2.86-7.14)	0.93(0.36-2.37)	0.56(0.074-4.15)	3.42(2.23-5.24)	3.04(1.99-4.63)	-
***0901**						
Frequency	0.12	0.01	0.031	0.087	0.08	0.026
P-value[a]	2.44×10^{-8}	0.325	0.868	7.55×10^{-6}	2.55×10^{-5}	-
OR (95% CI)[a]	4.82(2.64-8.82)	0.38(0.05-2.82)	1.19(0.16-8.99)	3.50(1.96-6.24)	3.21(1.82-5.69)	-
***030101**						
Frequency	0.1	0.02	0.031	0.083	0.077	0.043
P-value[a]	9.47×10^{-4}	0.283	0.748	0.01	0.0214	-
OR (95% CI)[a]	2.58(1.44-4.61)	0.47(0.11-1.94)	0.72(0.096-5.39)	2.01(1.17-3.46)	1.85(1.09-3.16)	-

a.Each subset versus controls b.Missing information of anti-topoisomerase I antibody status in 6 patients; Ab, antibody; CI, confidence interval

Table 3. Association of SNPs of HLA-*DPB1* and –*DPB2* with systemic sclerosis in Caucasians.

Alleles (Minor allele)	Systemic sclerosis[b]					Controls
	Anti-topoisomerase I Ab		Anti-centromere Ab		Total	
	(+) (n=183)	(-) (n=917)	(+) (n=316)	(-) (n=784)	(n=1,107)	(n=2,731)
rs3128930 (A)						
Frequency	0.31	0.28	0.3	0.28	0.29	0.27
P-value[a]	0.117	0.295	0.0564	0.47	0.14	-
OR (95% CI)[a]	1.20(0.95-1.51)	1.01(0.95-1.20)	1.19(1.00-1.43)	1.05(0.92-1.19)	1.09(0.97-1.21)	-
rs7764491 (C)						
Frequency	0.14	0.03	0.025	0.058	0.049	0.043
P-value[a]	$4.84x10^{-16}$	0.0175	0.036	0.0155	0.29	
OR (95% CI)[a]	3.56(2.57-4.94)	0.70(0.52-0.94)	0.58(0.35-0.97)	1.36(1.06-1.75)	1.14(0.90-1.44)	-
rs7763822 (T)						
Frequency	0.14	0.031	0.024	0.059	0.049	0.043
P-value[a]	$7.58x10^{-17}$	0.0214	0.0227	0.0076	0.236	-
OR (95% CI)[a]	3.65(2.64-5.04)	0.71(0.53-0.95)	0.55(0.32-0.93)	1.40(1.09-1.80)	1.15(0.91-1.46)	-
rs3128965 (A)						
Frequency	0.14	0.19	0.25	0.16	0.18	0.18
P-value[a]	0.0237	0.394	$3.20x10^{-5}$	0.01	0.99	-
OR (95% CI)[a]	0.70(0.52-0.96)	1.06(0.93-1.21)	1.50(1.24-1.82)	0.82(0.70-0.95)	1.00 (0.88-1.14)	-
rs3117230 (G)						
Frequency	0.17	0.26	0.29	0.22	0.24	0.24
P-value[a]	0.00765	0.055	$1.12x10^{-3}$	0.32	0.42	-
OR (95% CI)[a]	0.69(0.52-0.91)	1.13(1.00-1.28)	1.36(1.13-1.63)	0.93(0.81-1.07)	1.05(0.93-1.18)	-

a.Each subset versus controls b. Missing information of autoantibody status in 6 patients; Ab, antibody; CI confidence interval

Supplementary Table 1. Associaiton of rs312930, rs7764491 and rs3128965 with subsets of systemic sclerosis in Koreans.

SNPs	Systemic sclerosis				Controls
(Minor allele)	Skin subset				
	Limited (n=67)	Diffuse (n=66)	Limited/Cent+ (n=12)	Diffuse/Topo+ (n=45)	
rs3128930 (A)					
Frequency	0.33	0.39	0.13	0.49	0.15
P-value[a]	$1.67x10^{-7}$	$1.15x10^{-11}$	0.742	$3.33x10^{-16}$	
OR (95% CI)[a]	2.79(1.88-4.15)	3.59(2.44-5.29)	0.81(0.24-2.76)	5.46(3.50-8.51)	
rs7764491(C)					
Frequency	0.11	0.2	0.083	0.26	0.057
P-value[a]	0.0123	$5.28x10^{-10}$	0.576	$1.77x10^{-12}$	
OR (95% CI)[a]	2.10(1.16-3.81)	4.29(2.62-7.03)	1.52(0.35-6.59)	5.73(3.35-9.80)	
rs3128965 (A)					
Frequency	0.22	0.17	0.042	0.22	0.093
P-value[a]	$1.29x10^{-5}$	$3.68x10^{-3}$	0.387	$1.09x10^{-4}$	
OR (95% CI)[a]	2.68(1.70-4.24)	2.05(1.25-3.36)	0.42(0.056-3.16)	2.78(1.62-4.74)	

a.Each subset versus controls; Ab, antibody; Cent, anti-centromere antibody; CI confidence interval; Topo, anti-topoisomerase I antibody

Supplementary Table 2. Association of SNPs of HLA-DPB1 and –DPB2 with subsets of systemic sclerosis in Caucasians

SNPs	Systemic sclerosis				Controls
(Minor allele)	Skin subset				
	Limited	Diffuse	Limited/Cent+	Diffuse/Topo+	
	(n=654)	(n=419)	(n=277)	(n=88)	(n=2,731)
rs3128930 (A)					
Frequency	0.28	0.28	0.31	0.33	0.27
P-value[a]	0.238	0.376	0.041	0.072	-
OR (95% CI)[a]	1.08(0.94-1.24)	1.08(0.91-1.27)	1.22(1.01-1.48)	1.34(0.97-1.85)	-
rs7764491(C)					
Frequency	0.04	0.62	0.025	0.14	0.043
P-value[a]	0.647	0.014	0.0485	4.76×10^{-10}	-
OR (95% CI)[a]	0.93(0.68-1.27)	1.47(1.08-2.02)	0.58(0.34-1.00)	3.73(2.39-5.81)	-
rs7763822(T)					
Frequency	0.04	0.064	0.024	0.15	0.043
P-value[a]	0.632	0.007	0.0299	3.99×10^{-11}	-
OR (95% CI)[a]	0.93(0.68-1.26)	1.52(1.12-2.07)	0.54(0.31-0.95)	3.93(2.54-6.08)	-
rs3128965 (A)					
Frequency	0.2	0.16	0.26	0.13	0.18
P-value[a]	0.22	0.0765	1.71×10^{-5}	0.047	-
OR (95% CI)[a]	1.1(0.94-1.28)	0.84(0.69-1.02)	1.56(1.27-1.90)	0.63(0.40-1.00)	-
rs3117230(G)					
Frequency	0.26	0.21	0.3	0.15	0.24
P-value[a]	0.046	0.15	6.9×10^{-4}	6.08×10^{-3}	-
OR (95% CI)[a]	1.15(1.00-1.33)	0.88(0.73-1.04)	1.40(1.15-1.70)	0.55(0.36-0.84)	-

a.Each subset versus controls; Ab, antibody; Cent, anti-centromere antibody; CI confidence interval; Topo, anti-topoisomerase I antibody

Supplementary Table 3. Comparison of Caucasian SSc patients with and without autoantibodies to topo I or centromere protein.

Alleles	Anti-topoisomerase I Ab			Anti-centromere Ab		
(Minor allele)	(+) (n=183)	(-) (n=917)	p-values[a] OR(95% CI)	(+) (n=316)	(-) (n=784)	p-values[b] OR(95% CI)
rs3128930 (A)						
Frequency	0.31	0.28	0.33	0.30	0.28	0.212
			1.13(0.88-1.44)			1.14(0.93-1.39)
rs7764491 (C)						
Frequency	0.14	0.030	2.86×10^{-18}	0.025	0.058	1.5×10^{-3}
			5.11(3.42-7.63)			0.43(0.25-0.73)
rs7763822 (T)						
Frequency	0.14	0.031	8.61×10^{-19}	0.024	0.059	5.60×10^{-4}
			5.12(3.46-7.68)			0.39(0.22-0.68)
rs3128965 (A)						
Frequency	0.14	0.19	0.0116	0.25	0.16	1.07×10^{-7}
			0.66(0.48-0.91)			1.84(1.46-2.30)
rs3117230 (G)						
Frequency	0.17	0.26	7.35×10^{-4}	0.29	0.22	4.77×10^{-4}
			0.61(0.45-0.81)			1.45(1.18-1.79)

a. Comparison between anti-topoisomerase I antibody + and -; b. Comparison between anti-centromere antibody + and -.

Supplementary Table 4. Association of the SNPs with SSc patients with and without autoantibodies to DNA topoisomerase I and centromere protein in African Americans .

SNPs	Systemic sclerosis					Controls
(Minor allele)	Anti-topisomerase-I Ab		Anti-centromere Ab		Total	
	(+)	(-)	(+)	(-)		
	(n=9)	(n=61)	(n=5)	(n=65)	(n=70)	(n=90)
rs3128930 (A)						
Frequency	0.5	0.55	0.3	0.56	0.54	0.44
P-value[a]	0.615	0.0588	0.391	0.0325	0.0637	-
OR (95% CI)[a]	1.28(0.49-3.38)	1.56(0.98-2.48)	0.55(0.14-2.19)	1.64(1.04-2.59)	1.52(0.98-2.38)	-
rs7764491(C)						
Frequency	0.28	0.14	0.1	0.16	0.16	0.083
P-value[a]	9.05×10^{-3}	0.109	0.853	0.0297	0.036	-
OR (95% CI)[a]	4.23(1.33-13.48)	1.82(0.87-3.79)	1.22(0.14-10.31)	2.16(1.07-4.37)	2.09(1.04-4.19)	-
rs7763822(T)						
Frequency	0.28	0.14	0.1	0.16	0.16	0.083
P-value[a]	9.05×10^{-3}	0.121	0.853	0.0339	0.0405	-
OR (95% CI)[a]	4.23(1.33-13.48)	1.78(0.85-3.72)	1.22(0.14-10.31)	2.12(1.05-4.29)	2.05(1.02-4.12)	-
rs3128965 (A)						
Frequency	0	0.067	0	0.063	0.058	0.068
P-value[a]	0.253	0.959	0.393	0.844	0.713	-
OR (95% CI)[a]	-	0.98(0.39-2.47)	-	0.91(0.36-2.30)	0.84(0.33-2.12)	-
rs3117230(G)						
Frequency	0.28	0.41	0.2	0.41	0.39	0.38
P-value[a]	0.383	0.628	0.247	0.649	0.844	-
OR (95% CI)[a]	0.62(0.21-1.82)	1.12(0.70-1.80)	0.40(0.83-1.96)	1.11(0.70-1.77)	1.05(0.66-1.65)	-

a.Each subset versus controls; Ab, antibody; CI confidence interval

Supplementary Table 5. Association of the SNPs with SSc patients with and without autoantibodies to DNA topoisomerase I and centromere protein in Hispanics.

SNPs	Systemic sclerosis					Controls
(Minor allele)	Anti-topisomerase-I Ab		Anti-centromere Ab		Total	
	(+)	(-)	(+)	(-)		
	(n=17)	(n=44)	(n=12)	(n=49)	(n=61)	(n=90)
rs3128930(A)						
Frequency	0.29	0.38	0.29	0.37	0.35	0.24
P-value[a]	0.493	0.02	0.571	0.0236	0.0324	-
OR(95% CI)[a]	1.33(0.59-3.00)	1.91(1.10-3.33)	1.31(0.51-3.38)	1.85(1.01-3.17)	1.74(1.05-2.89)	-
rs7764491(C)						
Frequency	0.21	0.034	0	0.1	0.082	0.045
P-value[a]	$7.98x10^{-4}$	0.676	0.289	0.066	0.185	-
OR (95% CI)[a]	5.51(1.85-16.43)	0.75(0.19-2.90)	-	2.42(0.92-6.34)	1.90(0.73-4.95)	-
rs7763822(T)						
Frequency	0.21	0.034	0	0.01	0.082	0.044
P-value[a]	$7.21x10^{-4}$	0.688	0.292	0.0623	0.177	-
OR (95% CI)[a]	5.57(1.87-16.62)	0.76(0.20-2.93)	-	2.44(0.93-6.41)	1.92(0.74-5.01)	-
rs3128965(A)						
Frequency	0.029	0.2	0.25	0.13	0.16	0.16
P-value[a]	0.0429	0.381	0.278	0.532	0.904	-
OR (95% CI)[a]	0.16(0.020-1.20)	1.34(0.70-2.59)	1.73(0.63-4.77)	0.80(0.39-1.62)	0.96(0.51-1.82)	-
rs3117230(G)						
Frequency	0.088	0.35	0.25	0.29	0.28	0.21
P-value[a]	0.0972	0.013	0.656	0.159	0.173	-
OR (95% CI)[a]	0.36(0.11-1.26)	2.04(1.16-3.61)	1.25(0.46-3.38)	1.50(0.85-2.65)	1.45(0.85-2.48)	-

a.Each subset versus controls; Ab, antibody; CI confidence interval

Attenuation of fibrosis in vitro and in vivo with Sparc siRNA

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Attenuation of fibrosis *in vitro* and *in vivo* with Sparc siRNA

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ABSTRACT

OBJECTIVE: SPARC (SECRETED PROTEIN, ACIDIC AND RICH IN CYSTEINE) IS A MATRIC WHICH ALONG WITH OTHER EXTRACELLULAR MATRIX (ECM) COMPONENTS INCLUDING CO OVER-EXPRESSED IN FIBROTIC DISEASES. THE PURPOSE OF THIS STUDY WAS TO EXAMINE W SPARC CAN REGULATE COLLAGE EXPRESSION *in vitro* AND *in vivo*, AND SUBSEQUENTLY ATTENUATE FIBR STIMULATION BY BLEOMYCIN IN MOUSE SKIN AND LUNGS.

METHODS: IN *in vitro* STUDIES, SKIN FIBROBLASTS OBTAINED FROM KNOCK-IN MOUSE ($T\beta R1^{CA}$; CRE-ER) WERE TRANSFECTED WITH SPARC SIRNA. GENE AND PROTEIN EXPRESSIONS OF THE CTGF WERE EXAMINED BY REAL-TIME RT-PCR AND WESTERN BLOTTING, RESPECTIVELY. IN *in vivo* STUDIES, C57BL/6 MICE WERE INDUCED FOR SKIN AND LUNG FIBROSIS BY BLEOMYCIN AND FOLLOWE TREATMENT THROUGH SUBCUTANEOUS INJECTION AND INTRATRACHEAL INSTILLAT PATHOLOGICAL CHANGES OF SKIN AND LUNGS WERE ASSESSED BY HEMATOXYLIN AND EOS TRICHROME STAINS. THE EXPRESSION CHANGES OF COLLAGEN IN THE TISSUES WERE ASSE RT-PCR AND NON-CROSSLINKED FIBRILLAR COLLAGEN CONTENT ASSAYS.

RESULTS: SPARC SIRNA SIGNIFICANTLY REDUCED GENE AND PROTEIN EXPRESSION OF C FIBROBLASTS OBTAINED FROM $T\beta R1^{CA}$; CRE-ER MOUSE THAT WAS INDUCED FOR CONSTITUTIVEL TGF-β RECEPTOR I. SKIN AND LUNG FIBROSIS INDUCED BY BLEOMYCIN WAS MARKEDLY RED WITH SPARC SIRNA. THE ANTI-FIBROTIC EFFECT OF SPARC SIRNA *in vivo* WAS ACCOMPANIED BY AN INHIBITION OF CTGF EXPRESSION IN THESE SAME TISSUES.

CONCLUSION: SPECIFIC INHIBITION OF SPARC EFFECTIVELY REDUCED FIBROTIC CHANG *in vitro* AND *in vivo*. SPARC INHIBITION MAY REPRESENT A POTENTIAL THERAPEUTIC APPROACH TO FIBROTI

INTRODUCTION

FIBROSIS IS A GENERAL PATHOLOGICAL PROCESS IN WHICH EXCESSIVE DEPOSITIO MATRIX (ECM) OCCURS IN THE TISSUES. IT IS CURRENTLY UNTREATABLE. ALTHOUGH THE ANTI-INFLAMMATORY AND IMMUNOSUPPRESSIVE AGENTS SUCH AS COLCHICINES, I CORTICOSTEROIDS AND CYCLOPHOSPHAMIDE HAVE BEEN REPORTED, MANY OF THESE A PROVEN SUCCESSFUL (1-3). RECENTLY, SPARC (SECRETED PROTEIN, ACIDIC AND RICH MATRICELLULAR COMPONENT OF THE ECM, HAS BEEN REPORTED AS A BIO-MARKER FOR FIBROTIC DISEASES, SUCH AS INTERSTITIAL PULMONARY FIBROSIS, RENAL INTERSTITI ATHEROSCLEROTIC LESIONS AND SCLERODERMA OR SYSTEMIC SCLEROSIS (SSC) (4-9). N EXPRESSION OF SPARC HAS BEEN OBSERVED IN AFFECTED SKIN AND CIRCULATION OF (10,11), A DEVASTATING DISEASE OF SYSTEMIC FIBROSIS, AS WELL AS IN CULTURED D OBTAINED FROM SSC SKIN (8,9).

SPARC, ALSO CALLED OSTEONECTIN OR BM-40, IS AN IMPORTANT MEDIATOR C INTERACTION (12). INCREASING EVIDENCE INDICATES THAT SPARC MAY PLAY AN IMPORT FIBROSIS. IN ADDITION TO ITS HIGHER EXPRESSION LEVEL IN THE TISSUES OF FIBROTIC D SHOWN A CAPACITY TO STIMULATE THE TRANSFORMING GROWTH FACTOR BETA (TGF-β) SIGNALING SYSTEM (13). INHIBITION OF SPARC ATTENUATES THE PROFIBROTIC EFFECT OF EXOGENOUS TGF-β IN CULTURED HUMAN FIBROBLASTS (14). MOREOVER, IN ANIMAL STUDIES, SPARC-NULL MICE DISPLAY A DIMINI PULMONARY FIBROSIS COMPARED WITH CONTROL MICE AFTER EXPOSURE TO BLEOMYCIN ANTIBIOTIC WITH A PROFIBROTIC EFFECT (15). THESE OBSERVATIONS SUGGEST THAT SP BIO-TARGET FOR ANTI-FIBROTIC THERAPY. THE STUDIES DESCRIBED HERE AIMED TO EXP INHIBITION OF SPARC WITH SIRNA TO COUNTER FIBROTIC PROCESSES BOTH *in vitro* AND *in vivo* USING MURINE MODELS.

MATERIALS AND METHODS

Fibroblast cell lines from Tgfbr1 knock-in mouse. CONSTITUTIVELY ACTIVATED TGFBR1 M WHICH RECAPITULATED CLINICAL, HISTOLOGICAL, AND BIOCHEMICAL FEATURES OF HU REPORTED PREVIOUSLY (16). THEY ARE TERMED TBRI^{CA}; CKI-TBRI MICE AND HARBOR BOTH THE DNA FOR

INDUCIBLE CONSTITUTIVELY ACTIVE TGF β RECEPTOR I (TβRI) MUTATION TARGETED TO THE *ROSA26* LOCUS, AND A CRE-ER TRANSGENE DRIVEN BY A *Col1* FIBROBLAST-SPECIFIC PROMOTER. ADMINISTRATI 4-HYDROXYTAMOXIFEN (4-OHT) 2 WEEKS AFTER BIRTH ACTIVATES THE EXPRESSION OF CO TGFBR1 IN THE MICE (16). FIBROBLASTS WERE DERIVED FROM SKIN BIOPSY SPECIMENS OF CULTURES WERE MAINTAINED IN DMEM WITH 10% FCS AND SUPPLEMENTED WITH ANTIBI PENICILLIN AND 50 μG/ML STREPTOMYCIN). FIFTH-PASSAGE FIBROBLAST CELLS WERE SEEDED AT 10^5 CELLS IN 25-CM^2 FLASKS AND GROWN UNTIL CONFLUENCE. EXPERIMENTS WERE PERFORMED

Transient transfection with siRNA in fibroblasts. DOUBLE-STRANDED ON-TARGET *plus* SIRNAS OF MURINE *Sparc* AND *Ctgf* WERE PURCHASED FROM DHARMACON, INC. (LAFAYETTE, C CORRESPONDING TARGET SEQUENCES ARE 5'- GCACCACACGUUUCUUUG -3' FOR *Sparc* AND 5'- GCACCAGUGUGAAGACAUA -3' FOR *Ctgf*, RESPECTIVELY. THE CULTURE MEDIUM IN EACH C FLASK WITH CONFLUENT FIBROBLASTS WAS REPLACED WITH OPTI-MEM I MEDIUM (INVITRO WITHOUT FCS AND ANTIBIOTICS. THE FIBROBLASTS WERE INCUBATED FOR 24 HOURS AND TH SIRNA OR CTGF SIRNA IN A CONCENTRATION OF 100 NMOL/L, USING DHARMA*FECT*™ 1 SIRNA TRANSFECTION REAGENT (DHARMACON). FIBROBLASTS WITH NON-TARGETING SIRNA (DHAR WERE USED AS NEGATIVE CONTROLS. AFTER 24 HOURS, THE CULTURE MEDIUM WAS REPLAC CELLS TRANSFECTED WITH SIRNA WERE EXAMINED AFTER 72 HOURS OF TRANSFECTION AN PROTEIN EXPRESSION ANALYSIS. THE EXPERIMENTS WERE PERFORMED IN TRIPLICATES.

Animal models of fibrosis. C57BL/6 MICE OF ABOUT 20 GRAMS WERE PURCHASED F JACKSON LABORATORY (BAR HARBOR, MAINE). BLEOMYCIN FROM TEVA PARENTERAL MED CA) WAS DISSOLVED IN SALINE AND USED IN THE MICE AT A CONCENTRATION OF 3.5 UN FIBROSIS WAS INDUCED IN THESE MICE WITH ONCE INTRATRACHEAL INSTILLATION OF BL μL. FOR DERMAL FIBROSIS, FEMALE C57BL/6 MICE AT 6 WEEKS (WEIGHING ABOUT 20 G) WERE TREA WEEKS WITH LOCAL SUBCUTANEOUS INJECTION OF 100 μL BLEOMYCIN IN THE SHAVED LOWER BACK. FOUR WERE USED IN EACH GROUP. THE ANIMAL PROTOCOLS WERE APPROVED BY THE CENTER FO MEDICINE AND CARE IN THE UNIVERSITY OF TEXAS HEALTH SCIENCE CENTER AT HO INSTITUTIONAL ANIMAL USE AND CARE COMMITTEE OF M.D. ANDERSON CANCER CENTER.

Administration of siRNAs *in vivo*. FOR PULMONARY FIBROSIS, 5 μG OF SIRNA FOR *in vivo* USE (SISTABLE, DHARMACON), MIXED WITH DHARMA*FECT*™ 1 SIRNA TRANSFECTION REAGENT, WAS

ADMINISTRATED INTRATRACHEALLY IN 60 μL ON DAYS 2, 5, 12 AFTER BLEOMYCIN TREATMENT. ALL THE M

SACRIFICED ON DAY 23 AFTER ANESTHESIA, AND THE LUNG SAMPLES WERE COLLECTED. TH

BY 4% FORMALIN AND USED FOR FURTHER HISTOLOGICAL ANALYSIS. THE RIGHT LUNGS W

PIECES AND DIVIDED INTO 2 PARTS, ONE FOR RNA EXTRACTION AND ONE FOR COLLAGEN C

DERMAL FIBROSIS, THE ABOVE SIRNAS WERE INJECTED INTO THE SAME AREA AS THAT OF

AFTER BLEOMYCIN TREATMENT AND CONTINUED FOR 4 WEEKS. THE MICE WERE SACRIFIC

SKIN SAMPLES WERE COLLECTED. SALINE WAS USED AS NEGATIVE CONTROL IN BOTH FIBROSIS STUDIES.

Determination of gene expression by quantitative RT-PCR. TOTAL RNA FROM EACH CELL LINE WAS EXTRACTED FROM THE CULTURED FIBROBLASTS USING RNEASY MINI KIT (QIAGEN MICE LUNG AND SKIN TISSUES, THE MINCED SAMPLES WERE HOMOGENIZED IN LYSES SOLUT ST. LOUIS, MO) WITH A BLENDER. THEN TOTAL RNA WAS EXTRACTED USING GENELUTE™ M RNA MINIPREP KIT (SIGMA-ALDRICH). COMPLEMENTARY DNA (CDNA) WAS SYNTHESIZ MULTISCRIBE™ REVERSE TRANSCRIPTASE (APPLIED BIOSYSTEMS). QUANTITATIVE REAL-T PERFORMED USING AN ABI 7900 SEQUENCE DETECTOR SYSTEM (APPLIED BIOSYSTEMS, FOS THE SPECIFIC PRIMERS AND PROBES FOR EACH GENE (*Col1a2, Col3a1, Ctgf, and Sparc*) WERE PURCHASED FROM THE ASSAYS-ON-DEMAND PRODUCT LINE (APPLIED BIOSYSTEMS). SYNTHESIZED CDN WITH PRIMERS/PROBES IN 2 × TAQMAN UNIVERSAL PCR BUFFER AND THEN ASSAYED O SEQUENCE DETECTOR. THE DATA OBTAINED FROM THE ASSAYS WERE ANALYZED WIT (APPLIED BIOSYSTEMS). THE EXPRESSION LEVEL OF EACH GENE IN EACH SAMPLE WAS N *Gapdh* TRANSCRIPT LEVEL.

Western blot analysis. THE CELLULAR LYSATES EXTRACTED FROM THE CULTURED FI USED FOR PROTEIN ASSAYS. THE PROTEIN CONCENTRATION WAS DETERMINED BY A SPEC BRADFORD PROTEIN ASSAY KIT (BIO-RAD LABORATORIES, HERCULES, CA). EQUAL AMOUN EACH SAMPLE WERE SUBJECTED TO SODIUM DODECYL SULFATE-POLYACRYLAMIDE G RESOLVED PROTEINS WERE TRANSFERRED ONTO PVDF MEMBRANES AND INCUBATED WITH ANTIBODIES, INCLUDING ANTI-TYPE I COLLAGEN ANTIBODY (BIODESIGN INTERNATIONAL, ANTIBODY (GENETEX INC, SAN ANTONIO, TX), AND ANTI-SPARC ANTIBODY (R&D SYS MINNEAPOLIS, MN). MOUSE β-ACTIN (ALEXIS BIOCHEMICALS, SAN DIEGO, CA) WAS USED AS AN CONTROL. THE SECONDARY ANTIBODY WAS PEROXIDASE-CONJUGATED ANTI-RABBIT, AN

IGG. SPECIFIC PROTEINS WERE DETECTED BY CHEMILUMINESCENCE USING AN ENHANCED C SYSTEM (AMERSHAM, PISCATAWAY, NJ). THE INTENSITY OF THE BANDS WAS QUANTIFIED U SOFTWARE (MOLECULAR DYNAMICS, SUNNYVALE, CA).

Determination of collagen content. NON-CROSSLINKED FIBRILLAR COLLAGEN IN LUNG AND SKIN SAMPLES WAS MEASURED USING THE SIRCOL COLORIMETRIC ASSAY (BIOCOLOR, I TISSUES WERE HOMOGENIZED IN 0.5M ACETIC ACID WITH ABOUT 1:10 RATIO OF PEPSIN (S TISSUES WERE WEIGHTED, AND THEN INCUBATED OVERNIGHT AT 4°C WITH VIGOROUS STIRRING. DIGESTE SAMPLES WERE CENTRIFUGED AND THE SUPERNATANT WAS USED FOR THE ANALYSIS WITH THE PROTEIN CONCENTRATION WAS DETERMINED USING BRADFORD PROTEIN ASSAY KI CONTENT OF EACH SAMPLE WAS NORMALIZED TO TOTAL PROTEIN.

Histological analysis. BOTH THE TISSUE SAMPLES OF LUNG AND SKIN WERE FIXED IN 4% AND EMBEDDED IN PARAFFIN. SECTIONS OF 5 µM WERE STAINED EITHER WITH HEMATOXYLIN AND EOS AND MASSON'S TRICHROME.

Statistical Analysis. RESULTS WERE EXPRESSED AS MEAN ± SD. THE DIFFERENCE B DIFFERENT CONDITIONS OR TREATMENTS WAS ASSESSED BY STUDENTS'T-TEST. A P-VALUE CONSIDERED STATISTICALLY SIGNIFICANT.

RESULTS

Gene and protein expression of *Col1a2, Ctgf* and *Sparc* in the fibroblasts from TBR1CA; Cre-ER mice with and without transfection of siRNAs

AS MEASURED BY QUANTITATIVE REAL-TIME RT-PCR, *Col1a2*, *Ctgf* AND *Sparc* SHOWED INCREASED EXPRESSION IN THE FIBROBLASTS FROM TBR1^{CA}; CRE-ER MICE INJECTED WITH 4-OHT, IN WHICH TGFBR WAS CONSTITUTIVELY ACTIVE, COMPARED WITH THOSE IN THE TBR1^{CA}; CRE-ER MICE INJECTED WITH OIL (FIGURE 1). THE FOLD-CHANGES OF EACH GENE IN 4-OHT-INJECTED TGFBR1^{CA}; CRE-ER MICE FIBROBLASTS WERE 3.06 ± 1.42 FOR *Col1a2* (P = 0.050), 4.15 ± 1.18 FOR *Ctgf* (P = 0.049), AND 2.49 ± 0.63 FOR *Sparc* (P = 0.017), RESPECTIVELY. TO STUDY WHETHER INHIBITION OF SPARC INDUCED OF COLLAGEN IN THE FIBROBLASTS FROM CONSTITUTIVELY ACTIVE TGFBR1 MICE, WE TRA

INTO CULTURED FIBROBLASTS OBTAINED FROM TBRI^{CA}; COL-ER MICE INJECTED WITH 4-OHT. CTGF IS A DOWN-STREAM GENE IN THE TGF-β PATHWAY (17-20). INHIBITION OF CTGF REDUCED EXPRESSION FIBROTIC EFFECT OF TGF-β (21). WE USED CTGF SIRNA AS A POSITIVE CONTROL FOR INHIBITION OF COLLAGEN EXPRESSION. TRANSFECTION EFFICIENCY OF SIRNAS INTO FIBROBLASTS WA FLUORESCENT RNA DUPLEX *CLO* GREEN TRANSFECTION INDICATOR (DHARMACON) AND WAS DE BE OVER 80%. THE GENE EXPRESSION LEVELS FROM THE NON-TARGETING SIRNA TREATED COMPARED WITH THOSE FROM SALINE-TREATMENT FIBROBLASTS, AND NO SIGNIFICANT D (1.05 ± 0.18-FOLDS FOR *Col1a2*, 1.14 ± 0.16-FOLDS FOR *Ctgf*, AND 1.12 ± 0.12-FOLDS FOR *Sparc*). THEREFORE, IN THE FOLLOWING *in vitro* STUDY, FIBROBLASTS WITH NON-TARGETING SIRNA TREATM USED AS NEGATIVE CONTROLS. SEVENTY-TWO HOURS AFTER SPARC SIRNA OR CTGF SI SIGNIFICANT REDUCTIONS OF *Sparc* (95%) BY SPARC SIRNA AND *Ctgf* (64%) BY CTGF SIRNA WERE OBSERVED IN THE FIBROBLASTS (FIGURE 2A). IN PARALLEL, *Col1a2* SHOWED DECREASED E SIRNA TRANSFECTED FIBROBLASTS (27% AND 29% DECREASE WITH P < 0.05 FOR CTGF SIR SIRNA, RESPECTIVELY) (FIGURE 1). WESTERN BLOT ANALYSIS SHOWED A SIMILAR LEVEL O OF TYPE I COLLAGEN BY EITHER SPARC SIRNA OR CTGF SIRNA TREATMENT. AS ILLUSTRATE 2C, BOTH SPARC SIRNA AND CTGF SIRNA SHOWED SIGNIFICANT ATTENUATION OF COLLAG FIBROBLASTS (P = 0.009 OR 0.015, RESPECTIVELY). CTGF AND SPARC PROTEIN LEVELS ALSO BY SPARC SIRNA (P = 0.056 OR 0.0004, RESPECTIVELY) OR CTGF SIRNA TREATMENT (P = 0.002 O

siRNAs ameliorated fibrosis in skin and reduced inflammation in lungs induced by bleomycin

HE STAINS OF MOUSE SKIN TISSUES (FIGURE 3) SHOWED THAT 4-WEEK INJECTIONS OF BL SIGNIFICANT FIBROSIS IN SKIN WHERE THE FAT CELLS WERE REPLACED BY FIBER BUN COMPARED WITH NORMAL SKIN INJECTED WITH SALINE ONLY (FIGURE 3.). BLEOMYCIN-IN WITH CTGF SIRNA OR SPARC SIRNA SHOWED THAT MOST OF THE FAT CELLS STILL EXIS WITHOUT PROMINENT FIBER BUNDLES (FIGURE 3.). MASSON'S TRICHROME STAINING OF SHOWED THE SAME RESULTS. NOTABLY, INCREASED HAIR FOLLICLES WERE INCONSISTENT AND SPARC SIRNA-TREATED BLEOMYCIN-INDUCED SKINS.

HE STAIN OF MOUSE LUNG TISSUES (FIGURE 4) SHOWED A SIGNIFICANT DISRUPTION OF THE INFILTRATION OF INFLAMMATORY CELLS IN THE LUNGS INDUCED BY BLEOMYCIN (FIGU TREATMENT WITH CTGF SIRNA OR SPARC SIRNA, THE DISRUPTION OF THE ALVEOLI WAS IN

INFILTRATING INFLAMMATORY CELLS (FIGURE 4.).

siRNAs attenuated over-expression of collagen and other fibrotic ECM genes induced by bleomycin in skin and lung tissues

BLEOMYCIN INJECTION INDUCED AN UP-REGULATION OF THE *Col1a2, Col3a1, Ctgf* AND *Sparc* IN BOTH SKIN (FIGURE 5A, P = 0.028, 0.016, 0.049 AND 0.0005, RESPECTIVELY) AND LUNG TISSUES (FIGUR 0.015, 0.005, 0.041 AND 0.056, RESPECTIVELY) OF THE MICE. HOWEVER, IN CTGF SIRNA OR SPA TREATED MICE SKIN THAT ALSO RECEIVED BLEOMYCIN INJECTION, THE EXPRESSION OF TH *Col1a2* AND *Col3a1* APPEARED TO BE NORMAL IN SKIN TISSUES (FIGURE 5A, P = 0.025 AND 0.003 FOR EACH GENE TREATMENT, AND P = 0.031 AND 0.010 IN SPARC SIRNA TREATMENT), AND WERE SIGNIFICAN LUNG TISSUES (ABOUT 2.7-FOLD REDUCTION FOR *Col1a2* AND 1.9-FOLD REDUCTION FOR *Col3a1*, COMPARED TO BLEOMYIN-INJECTED MICE WITHOUT SIRNA TREATMENT, P < 0.05 FOR BOTH) (FIGURE 5 B). COLLAGEN GENE EXPRESSION, THE *Ctgf* AND THE *Sparc* EXPRESSION WERE SIGNIFICANTLY REDUCED BY SIRNA AND CTGF SIRNA TREATMENT, RESPECTIVELY (FIGURE 5B). IN DETAIL, C BLEOMYCIN-INDUCED SKIN AND LUNGS, SPARC SIRNA NORMALIZED *Ctgf* EXPRESSION IN BOTH SKIN AND LUN (2.6-FOLD REDUCTION IN BOTH WITH P = 0.1 AND 0.039, RESPECTIVELY). SIMILARLY, CT REDUCED *Sparc* EXPRESSION IN SKIN AND LUNGS (2.9-FOLD AND 1.5-FOLD REDUCTION WITH P 0.188, RESPECTIVELY).

siRNAs reduced the collagen contents in bleomycin-induced mouse skin and lung samples: TO FURTHER EVALUATE ANTI-FIBROTIC EFFECTS OF SIRNAS ON THE FIBROGENESIS OF SKIN A CONTENT, A KEY MARKER IN ASSESSING COLLAGEN DEPOSITION, WAS MEASURED IN THE PULMONARY SAMPLES. QUANTIFICATION OF TOTAL COLLAGEN IN SKIN SAMPLES WITH THE 2.2-FOLD INCREASE IN BLEOMYCIN-INDUCED SKIN COMPARED WITH SALINE-INJECTED SKIN SIRNA TREATMENT REDUCED THE COLLAGEN CONTENT TO 47.6% (P = 0.03) OF THAT IN BL SKIN, AND SPARC SIRNA TREATMENT REDUCED THE COLLAGEN CONTENT TO 64.6% (P 6A).THERE WAS NO SIGNIFICANT DIFFERENCE OF COLLAGEN REDUCTION (P = 0.08) BETWE TREATMENT AND CTGF SIRNA TREATMENT.

THE SIRNA TREATMENTS ALSO SHOWED A REDUCTION OF COLLAGEN IN THE BLEOMYCIN-INDUCED MICE (FIGURE 6B). IN BLEOMYCIN-INDUCED MICE, COLLAGEN CONTE

WAS 3.6-FOLD HIGHER THAN THAT IN CONTROL MICE (SALINE-INJECTED MICE, P = 0.0135). IN SPARC SIRNA TREATED MICE THAT ALSO WERE BLEOMYCIN-INDUCED, COLLAGEN CONTEN 68% (P = 0.128) OR 58% (P = 0.019) OF THAT IN BLEOMYCIN-INDUCED MICE WITHOUT SIRNA T NO SIGNIFICANT DIFFERENCE OF COLLAGEN CONTENT WAS FOUND BETWEEN SPARC SIRNA SIRNA TREATMENT IN BLEOMYCIN-INJURED LUNGS (P = 0.28).

DISCUSSION

ALTHOUGH FIBROSIS IS USUALLY AN IRREVERSIBLE PATHOLOGICAL CONDITION, TARGETI EFFECTORS MAY REVERSE AN ACTIVE STATUS OF THE FIBROTIC PROCESS, AND SUBSEQUEN TGF-β SIGNALING PATHWAY IS ASSOCIATED WITH ACTIVE FIBROSIS (17). IT BEGINS WITH T TGF-β LIGAND TO THE TGF-β TYPE II RECEPTOR, WHICH CATALYSES THE PHOSPHORYLATION O RECEPTOR ON THE CELL MEMBRANE. THE TYPE I RECEPTOR THEN INDUCES THE PH RECEPTOR-REGULATED SMADS (R-SMADS) THAT BIND THE COSMAD. THE PHOSP R-SMAD/COSMAD COMPLEX ENTERS THE NUCLEUS ACTING AS TRANSCRIPTION FACTORS GENE EXPRESSION (18). CTGF (CONNECTIVE TISSUE GROWTH FACTOR) IS A DOWN-STREAM ACTIVATED BY THE TGF-β SIGNALING PATHWAY (19). ACTIVATION OF CTGF IS ASSOCIATED WITH PERSISTENT FIBROTIC CHANGES IN THE TISSUES, WHICH IS TYPICALLY REPRESENTED AS ACC COMPONENTS INCLUDING COLLAGENS (20).

THE STUDIES DESCRIBED HERE UTILIZED THE FIBROBLASTS OBTAINED FROM THE TBR1^{CA}; CRE-ER MICE THAT WERE INDUCED FOR CONSTITUTIVELY ACTIVE TGF-β RECEPTOR I. AFTER TRANSFECTION OF SPARC SIRN FIBROBLASTS SHOWED A DECREASED EXPRESSION OF COL1A2 THAT WAS ORIGINALLY O TBR1^{CA}; CRE-ER MICE (FIGURE 2). THIS PHENOMENON SUGGESTS THAT SPARC INHIBITION MA FIBROTIC TGF-β SIGNALING. ALTHOUGH THE SPECIFIC MECHANISM FOR THIS SUPPRESSION IS U PREVIOUS STUDIES HAVE DEMONSTRATED A MUTUAL REGULATORY RELATIONSHIP BETW SIGNALING (14,21-23). THIS NOTION ALSO IS SUPPORTED BY THE OBSERVATION OF AN OV SPARC IN THE FIBROBLASTS OF THE TBR1^{CA}; CRE-ER MICE (FIGURE 1). IT SHOULD BE NOTED THAT THE EXPRESSION IN THE FIBROBLASTS WAS NOT REDUCED UPON SPARC INHIBITION. THESE CONTRADICT OUR PREVIOUS REPORT OF PARALLEL INHIBITION OF SPARC AND CTGF EXP FIBROBLASTS BY SPARC SIRNA (14). A POSSIBLE EXPLANATION IS THAT OVER-EXPRESS

CONSTITUTIVELY ACTIVATED TGF-β SIGNALING IN THESE FIBROBLASTS MAY CONFER RESISTA DOWN-REGULATORY EFFECT FROM SPARC SIRNA. HOWEVER, SUCH RESISTANCE APPEAR INFLUENCE ON ANY DOWN-REGULATORY EFFECT OF SPARC SIRNA ON COLLAGEN TYPE 1, CTGF IS NOT A SOLE CONTRIBUTOR TO TGF-β SIGNALING-ASSOCIATED FIBROSIS.

BLEOMYCIN INDUCED FIBROSIS IN MICE USUALLY OCCURS AFTER INFLAMMATION IN β IS UP-REGULATED (24). OUR *in vivo* APPLICATION OF SPARC SIRNA DEMONSTRATED THAT INHIBITIO SIGNIFICANTLY REDUCED INFLAMMATION AND FIBROSIS IN SKIN AND LUNGS INDUCED B TREATMENT OF SKIN FIBROSIS, SPARC SIRNAS REDUCED FIBER BUNDLES ACCUMULATED IN MONONUCLEAR CELL INFILTRATES (FIGURE 3). IN ADDITION TO HISTOLOGICAL CHANG BLEOMYCIN-INDUCED SKIN TREATED WITH SPARC SIRNA SHOWED OVER 50% REDUCTION WITHOUT SPARC SIRNA TREATMENT (DATA NOT SHOWN). THE CHANGES OF TISSUE FIBROT CONFIRMED WITH SIGNIFICANTLY DECREASED COLLAGEN GENE EXPRESSION (FIGURE 5A FIBRILLAR COLLAGEN IN THE SKIN TISSUES ALSO SHOWED AN AVERAGE OF 35.4% REDUCTI TREATMENT (FIGURE 6A).

IN THE TREATMENT OF LUNGS, SPARC SIRNA REDUCED THE DISRUPTION OF THE ALVEOLI IN (FIGURE 4), WHICH WAS ACCOMPANIED WITH ATTENUATED GENE EXPRESSION AND PR COLLAGENS AS COMPARED TO THAT WITHOUT SIRNA TREATMENT (FIGURE 5B AND 6B). HOW HISTOLOGICAL EXAMINATIONS AND THE CHANGED LEVELS OF COLLAGEN GENES AND PROT APPEARED TO BE LESS PERFECT AS COMPARED TO THAT IN NORMAL CONTROL MICE. ON T HIGHER LEVELS OF GENE EXPRESSION OF *Col1A2* AND *Col3A1*, AND PROTEIN CONTENT OF COLLAGEN W OBSERVED IN BLEOMYCIN-INDUCED LUNG TISSUES WHEN THEY WERE COMPARED TO T (5.2-FOLD, 6.7-FOLD AND 3.6-FOLD INCREASE VS. 3.8-FOLD, 2.8-FOLD AND 2.2-FOLD INCREAS WHICH SUGGESTED THAT TISSUE DAMAGE AND FIBROSIS IN LUNG MIGHT BE MORE SEVERE THIS CASE, TREATMENT OF BLEOMYCIN-INDUCED LUNG DAMAGE MIGHT PRESENT A BIGGE OF SKIN, AND THE SIRNA TREATMENT THROUGH INTRATRACHEAL INSTILLATION MAY B OPTIMIZATION. THESE NOTIONS WERE SUPPORTED BY SIMILAR FINDINGS IN THE TREATM SIRNA, A POSITIVE CONTROL FOR ANTI-FIBROTIC EFFECTS.

NEVERTHELESS, SPARC INHIBITION SHOWED A CLEAR ANTI-FIBROTIC EFFECT IN BLEOMYC LUNG TISSUES. NOTABLY, THESE CHANGES WERE ACCOMPANIED WITH A SIGNIFICANT DOW *Ctgf* THAT PARALLELED WITH *Sparc* UP-REGULATION IN BLEOMYCIN-INDUCED TISSUES. THESE OBS COMBINING WITH THE RESULTS OF ANTI-FIBROTIC EFFECTS OF SPARC SIRNA IN FIBROBL KNOCK-IN MOUSE FURTHER SUPPORT A MUTUALLY REGULATORY RELATIONSHIP BETWE SIGNALING.

IN SUMMARY, STUDIES DESCRIBED HERE CONSISTENTLY DEMONSTRATED THAT INHIBITION SIGNIFICANTLY REDUCED COLLAGEN EXPRESSION IN BOTH *in vitro* TRANSGENIC TGFBR1 FIBROBLAST MODEL A *in vivo* BLEOMYCIN-INDUCED FIBROTIC MOUSE MODEL. THIS IS A FIRST ATTEMPT TO EXAMIN EFFECTS OF SPARC INHIBITION IN SKIN AND LUNG *in vivo*. THE RESULTS OBTAINED FROM THESE STU PROVIDE FAVORABLE EVIDENCE THAT SPARC MAY BE USED AS A BIO-TARGET FOR APPLICA THERAPIES.

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Figure legend

Figure 1. COMPARISON OF GENE EXPRESSION BETWEEN THE FIBROBLASTS OF TGFBR1^{CA}; CRE-ER MICE INJECTED WITH OIL AND 4-OHT. THE EXPRESSION LEVEL OF EACH GENE IN THE^{CA}; FIBROBL CRE-ER MICE INJECTED WITH OIL WAS NORMALIZED TO 1. BARS SHOW THE MEAN ± SD RESU 3 INDEPENDENT EXPERIMENTS PERFORMED IN TRIPLICATE. *, $p < 0.05$.

Figure 2. GENE AND PROTEIN EXPRESSION IN ORIGINAL AND SIRNA TREATED FIBROBLASTS CRE-ER MICE INJECTED WITH 4-OHT. (A) RELATIVE TRANSCRIPT LEVELS OF COL1A2, CTGF, EXPRESSION LEVEL OF EACH GENE IN THE FIBROBLAST LINES WITH NON-TARGETING SI TREATMENT WAS NORMALIZED TO 1. *, P < 0.05. (B) WESTERN BLOT ANALYSIS OF TYPE I COL AND SPARC IN THE FIBROBLASTS FROM CONSTITUTIVELY ACTIVE TGFBR1 MICE, WITH C SIRNA TRANSFECTION. N = NON-TARGETING SIRNA TREATMENT; C = CTGF SIRNA TREATM SIRNA TREATMENT. (C) DENSITOMETRIC ANALYSIS OF WESTERN BLOTS FOR PROTEIN LEVE SPARC. COMPARED TO NON-TARGETING SIRNA TREATMENT, CTGF SIRNA OR SPARC SIRNA T SHOWED SIGNIFICANT REDUCTION OF COL1 (P = 0.015 OR 0.009 RESPECTIVELY), CTGF (P =0. RESPECTIVELY) AND SPARC (P = 0.355 OR 0.0004, RESPECTIVELY).

Figure 3. REPRESENTATIVE HISTOLOGICAL ANALYSIS OF HE AND TRICHROME STAIN OF DIFFERENT TREATMENTS FOR 4 WEEKS IN LOW (4 X) AND HIGH MAGNIFICATIONS (20 X). SALINE (NEGATIVE CONTROL) ONLY; B. INJECTION WITH BLEOMYCIN ONLY; C: INJECTION TREATMENT WITH SPARC SIRNA; D. INJECTION WITH BLEOMYCIN AND TREATMENT WITH CT

Figure 4. REPRESENTATIVE HISTOLOGICAL FEATURES OF HE AND TRICHROME STAIN OF MO WITH DIFFERENT TREATMENTS INTRATRACHEALLY IN LOW (4 X) AND HIGH MAGNIFICATIO WITH SALINE (NEGATIVE CONTROL) ONLY; B. INJECTION WITH BLEOMYCIN ONLY ON DAY BLEOMYCIN ON DAY 0 AND SPARC SIRNA ON DAYS 2, 5, AND 12; D. INJECTION WITH BLEOMY AND CTGF SIRNA ON DAYS 2, 5, AND 12.

Figure 5. GENE EXPRESSION IN SKIN OR LUNG SAMPLES WITH DIFFERENT TREATMENTS. (A) RELATIVE TRANSCRIPT LEVELS OF *Col1a2, Col3a1, Ctgf,* AND *Sparc* IN SIRNA-TREATED OR UNTREATE BLEOMYCIN-INDUCED SKINS OR LUNGS, RESPECTIVELY. THE EXPRESSION LEVEL OF EACH LUNG SAMPLE FROM SALINE TREATED MICE WAS NORMALIZED TO 1. *, P < 0.05.

Figure 6. COLLAGEN CONTENTS IN SKIN (A) OR LUNG (B) SAMPLES WITH DIFFERENT TREATM CONTENT IN THE SKIN OR LUNG SAMPLE FROM SALINE TREATED MICE WAS NORMALIZED SALINE; B: BLEOMYCIN; B + C: BLEOMYCIN AND CTGF SIRNAS; B + S: BLEOMYCIN AND SPAR < 0.05.

Figure 1.

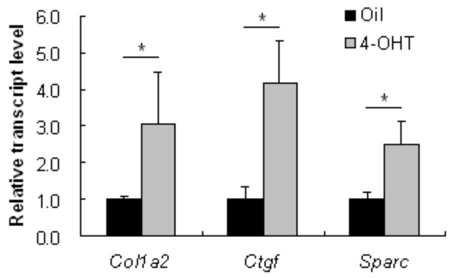


Figure 2.

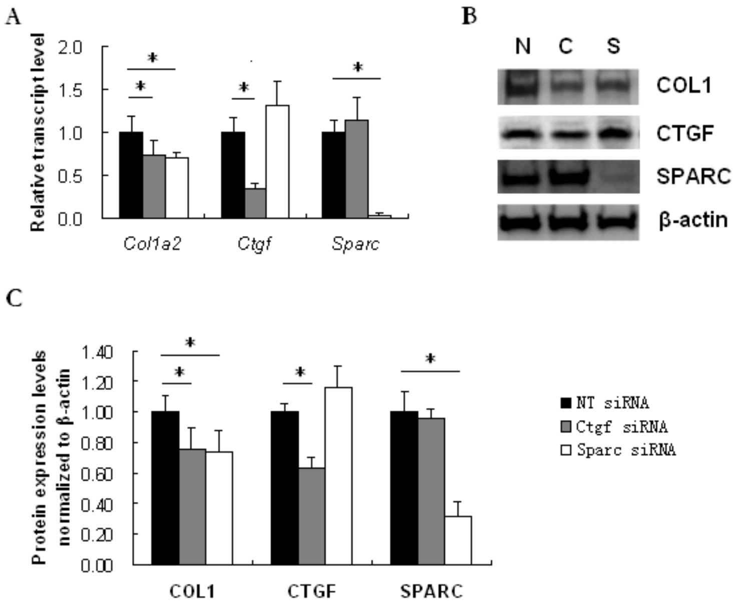


Figure 3.

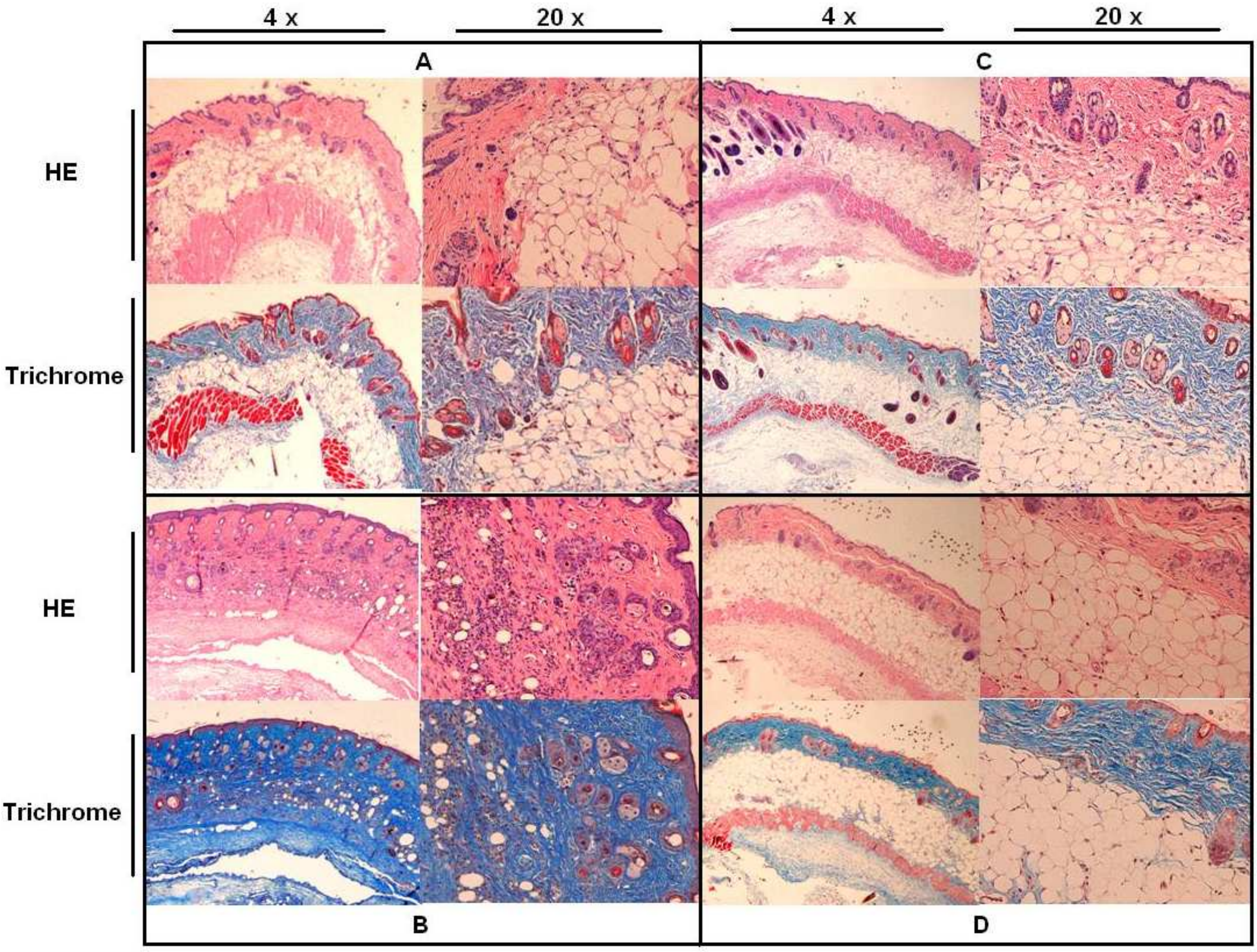


Figure 4.

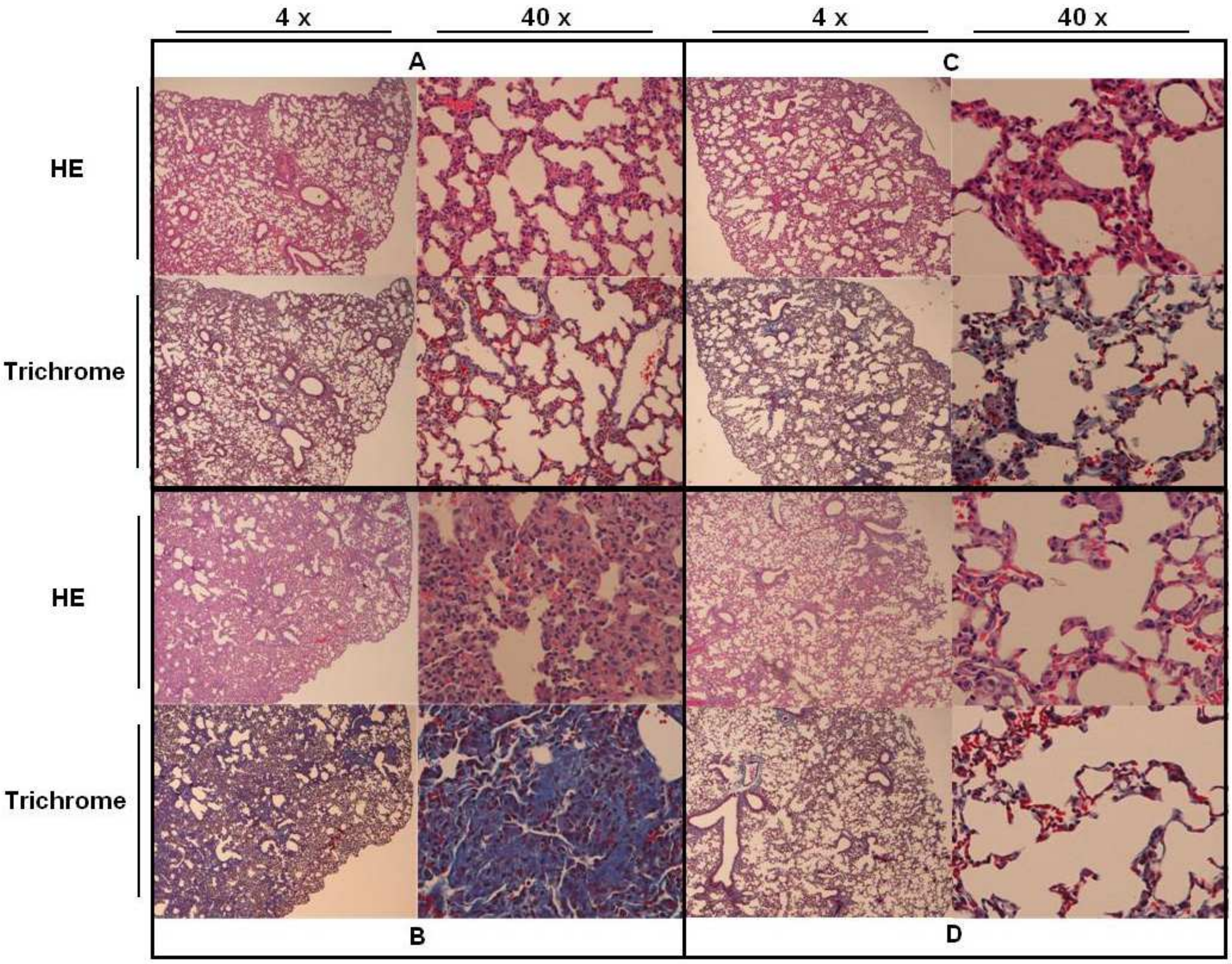


Figure 5.

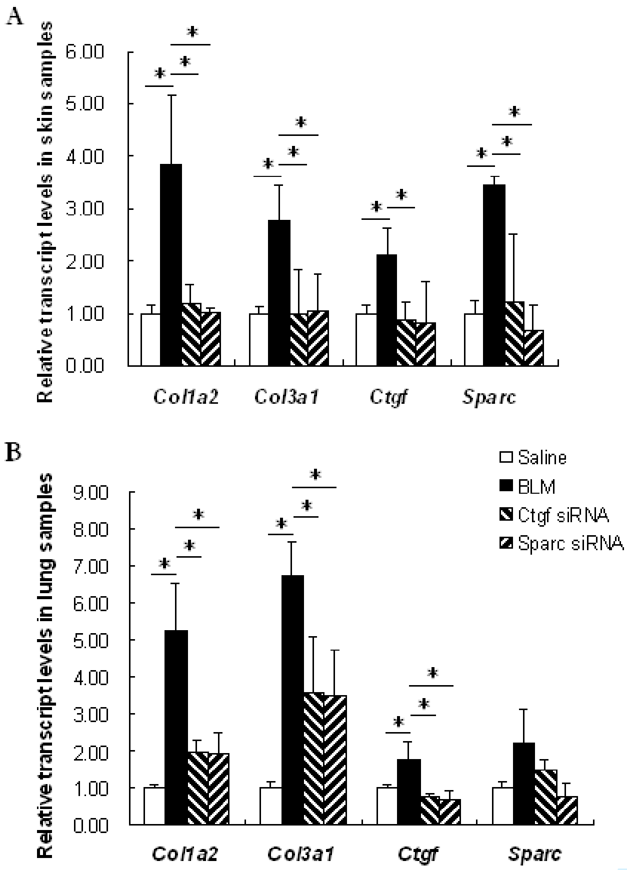
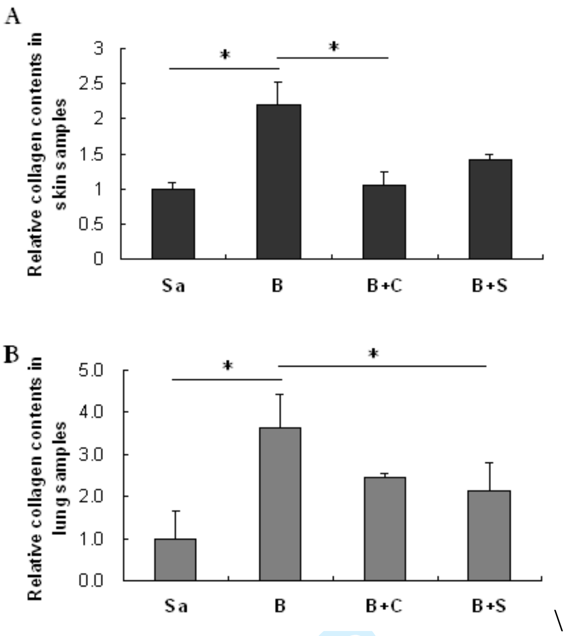

A
Relative transcript levels in skin samples
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Col1a2
Col3a1
Ctgf
Sparc
B
Relative transcript levels in lung samples
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Saline
BLM
Ctgf siRNA
Sparc siRNA
Col1a2
Col3a1
Ctgf
Sparc

Figure 6.


A
Relative collagen contents in skin samples
3
2.5
2
1.5
1
0.5
0
*
*
Sa
B
B+C
B+S
B
Relative collagen contents in lungs samples
5.0
4.0
3.0
2.0
1.0
0.0
*
*
Sa
B
B+C
B+S

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